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The Strange Story of *Fucus spiralis*

MESTRADO EM BIOLOGIA MARINHA

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ABSTRACT

Phylogeography has been a growing field since the 1970's. The evolutionary histories of both terrestrial and marine biota have been reconstructed in the last years and the North Atlantic has been a major target study area. Here we examined 733 individuals of the amphi-Atlantic brown seaweed *Fucus spiralis* from 21 populations covering its distributional range using a mitochondrial intergenic spacer and several EST-derived microsatellite loci. Our data recovered three *F. spiralis* entities based on their multilocus genotypes: one mainly in its southern range (*F. spiralis* South) where it is allopatric with *F. vesiculosus*, and two (*F. spiralis* Low and *F. spiralis* High) in northern populations where these species are sympatric. Using Single Strand Conformation Polymorphism (SSCP), a total of 16 mtDNA haplotypes were identified that separate the South (*F. spiralis* South) and North (*F. spiralis* Low and *F. spiralis* High) entities into two clades. The North clade haplotypes are shared, or similar, to the ones found in a parallel *F. vesiculosus* study but not the South clade ones. An agreement between markers was observed: distinct entities based on nuclear genotypes correspond in general to distinct mtDNA haplotypes and clades. Despite the parapatric occurrence of *F. spiralis* Low and *F. spiralis* High in northern populations, the lack of intermediate genotypes suggests reproductive isolation and an ongoing speciation process. We propose that the separation between Southern and Northern types is due to a long period of isolation in two geographical regions, likely to have been originated from two distinct glacial refugia: one in the southern range and another in the Brittany-North Iberia area. Furthermore, results suggest that western Atlantic and scattered Pacific populations were recolonized from a unique source of *F. spiralis* High.

Keywords: *Fucus spiralis*, hybridization, microsatellites, mitochondria, phylogeography, speciation

RESUMO

Desde a década de 70, que a filogeografia é uma área em ascensão. A história evolutiva de organismos terrestres e marinhos tem sido reconstruída nos últimos anos, sendo o Atlântico Norte uma das principais áreas de estudo. Neste estudo, examinaram-se 733 indivíduos de *Fucus spiralis* (alga castanha anfiatlântica), de 21 populações abrangendo todo o seu limite de distribuição, usando um *"intergenic spacer"* mitocondrial e vários microsatélites derivados de EST's. Através dos nossos dados determinaram-se três entidades distintas de *Fucus spiralis* baseados nos seus genótipos multilocus: um presente maioritariamente no seu limite de distribuição Sul (*F. spiralis* South) onde é alopátrico com *F. vesiculosus*, e dois (*F. spiralis* Low e *F. spiralis* High) em populações do Norte onde estas espécies se encontram em simpatria. Identificaram-se16 haplótipos mitocondriais através da técnica SSCP (Single Strand Conformation Polymorphism). Estes haplótipos permitem separar as entidades do Sul (*F. spiralis* South) e o do Norte (*F. spiralis* Low e *F. spiralis* High) em duas clades distintas. Os haplótipos encontrados na clade do Norte, são partilhados ou similares com aqueles encontrados num estudo paralelo feito com *F. vesiculosus*, contrariamente ao que se verifica na clade do Sul. Foi, também, observada uma concordância entre marcadores: entidades consideradas distintas com base em genótipos nucleares, correspondem a distintos haplótipos mitocondriais, assim como a diferentes clades. Apesar da parapatria existente entre *F. spiralis* Low e *F. spiralis* High nas populações do Norte, a inexistência de genótipos intermédios sugere um isolamento reprodutor e, quiçá, um processo de especiação em progresso. Desta forma, propomos que a separação entre os tipos do Sul e do Norte, seja devida a um longo período de isolamento em duas regiões geográficas provavelmente originadas a partir de dois refúgios glaciais distintos: um no seu limite de distribuição Sul e outro em toda a área que abrange o Norte da Ibéria e da Bretanha. Adicionalmente, os resultados sugerem que as populações do Atlântico Oeste e as escassas populações do Pacífico foram colonizadas a partir de uma única fonte de *F. spiralis* High.

Palavras-chave: Especiação, filogeografia, *Fucus spiralis*, hibridação, microsatélites e mitocondria.

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INTRODUCTION

Earth has been continuously exposed to cyclic climate fluctuations that are experienced differently in the various ecosystems from different parts of the globe, varying according to their regional characteristics (Hewitt, 2000). These events have undeniable effects on population structure, demography, and genetics and in their evolutionary history.

The last most striking episode of glaciation, the Last Glacial Maximum (LGM, *c.*21000 years BP), left the Northern Hemisphere covered with large ice sheets down to latitudes around 50º N (Clark & Mix, 2002). Populations were forced to adjust to this changing scenario and they would have had to either retreat southwards to ice-free refugia, or would have to face extinction. That is why lower latitudes often present higher genetic and allelic diversity supporting the idea of southward refugia sources through several Ice Ages that recolonized the north as the ice retreated, a pattern that Hewitt (2001) named as: "southern richness to northern purity". This historical dynamics of geographical contractions and expansions of species is since the mid 1970's the question posed by phylogeography. Phylogeography as an interdisciplinary field tries to view the big picture combining geographical history of species with its phylogeny, demography and ethology (Avise, 1998).

Marine biota in the North Atlantic were extremely affected by the LGM, an episode that lead to a sea level decrease between 120 and 135 m (Clark & Mix, 2002) and inevitably changed coastlines (Hewitt, 2000). Nevertheless, there is evidence that the intertidal habitat of the Northwest Atlantic was more greatly devastated during this event than the East, the eastern populations having been responsible for later recolonization as the glaciation ended (Wares & Cunningham, 2001). But not every European coast was suitable to survival and several Northeast Atlantic marine refugia have been proposed: the Iberian Peninsula is widely recognized as a terrestrial refugium (Hewitt, 2004) and has also been proposed as a marine one for both fauna (green crab (Roman & Palumbi, 2004), thornback ray (Chevolot et al., 2006), common goby (Gysels et al., 2004) and flora (the brown seaweed *F. serrauts*, Hoarau et al, 2007b). The English Channel area is a very interesting area due to the Hurd Deep depression that during the LGM may have remained marine, for seagrasses, polychaetes and red and brown seaweeds (Olsen et al., 2004; Provan et al., 2005; Jolly et al., 2006; Hoarau et al., 2007b); the ice-free Southwestern Ireland (Lambeck et al.,

2002) for both red and brown seaweeds (Provan et al., 2005; Jolly et al., 2006; Hoarau et al., 2007b) and bryozoan (Gómez et al., 2007); and the Azores, that are believed to have been little affected by the LGM (Rogerson et al., 2004) for thornback rays (Chevolot et al., 2006).

The Model Taxa

The genus *Fucus* is a common and dominant component of tidal rocky shores in north Atlantic (Lüning, 1990). This monophyletic genus comprises two major lineages (Serrão et al., 1999; Coyer et al., 2006) that share the characteristic of having both hermaphroditic and dioecious species. Interestingly both hermaphroditic species, *F. distichus* and *F. spiralis*, that belong to different lineages are also present in the North Pacific (Lüning, 1990). However, the discrete few populations of *F. spiralis* are regarded as a human introduction (Norris, 1974; Lüning, 1990; Serrão et al., 1999). Although a Pacific origin of *F. spiralis* has been proposed (Coyer et al., 2006), this hypothesis would make the species non-monophyletic, implying a taxonomic revision, as Atlantic *F. spiralis* would share a common ancestor with other *Fucus* species that was not shared with Pacific conspecifics. Understanding the phylogeographic history of this species with wide latitudinal and amphi-Atlantic distribution is the main question raised in this thesis.

Besides the recently detected *F. spiralis* of the Pacific, the lineage of *F. spiralis* includes only Atlantic taxa, all closely related and undistinguishable based on DNA sequences so far (Serrão et al., 1999; Coyer et al., 2006) but clearly separated based on microsatellite data (Billard et al., 2005a). One of these sister species, *F. vesiculosus*, a dioecious species, has a geographic distribution that strongly overlaps with *F. spiralis* (Lüning, 1990), although locally they are segregated: *F. vesiculosus* is found either at mid-low intertidal rocky shores or estuarine habitats whereas the hermaphroditic *F. spiralis* inhabits mainly the mid-high intertidal and is less common inside estuaries, although they occur together in contact zones between those typical habitats. In the southern range however, there are no sympatric populations of *F. vesiculosus* and *F. spiralis*, they are only found in estuaries and coastal environments, respectively (Ladah et al., 2003), and these allopatric populations are extremely differentiated from the remaining ones (Perrin et al., in prep).

Despite their contrasting mating systems and distinct intertidal zonation these two species are not reproductively isolated and are able to hybridize (Engel et al., 2003; Wallace et al., 2004; Billard et al., 2005b; Perrin et al., 2007). In fact more than one entity of *F. spiralis* have been described a century ago based on phenotypic and morphological variation: *F. spiralis* var *typicus* (Böergesen, 1909) and *F. spiralis* var *platycarpus* (Thuret) Batters 1902, both cited in Hamel (1939), and continue to receive recent attention (e.g., Scott et al., 2001; Garreta, 2001). Genetic approaches using microsatellites show the co-existence of distinct parapatric entities that have been described as '*F. spiralis* Low', the entity that shares the mid-high intertidal with *F.vesiculosus* and '*F. spiralis* High' the one standing by itself on the high shore (Billard, 2007), a differentiation already shown in the results of Engel et al. (2005), although not described as distinct entities there but rather as parapatric (High, *F. spiralis* only zone) and sympatric (Low, zone mixed with *F. vesiculosus*) populations, where the sympatric (Low shore) ones were very distinct from the parapatric ones (High) in Portugal but not so in North France (Engel et al., 2005).

Selection of Genetic Markers

Genetic Markers Used in Fucus

Several studies have been conducted to learn more about this challenging group of fucoid algae using different types of genetic markers such as ITS (Serrão et al., 1999), microsatellites (Wallace et al., 2004; Billard et al., 2005ab; Coleman & Brawley, 2005; Engel et al., 2005; Tatarenkov et al., 2005; Coyer et al., 2006; Perrin et al., 2007) mitochondrial DNA (mtDNA) (Coyer et al., 2006; Hoarau et al., 2007b) and chloroplast DNA (Coyer et al., 2002; Billard, 2007). However, each marker gives better or worst answers depending on the question raised. For example, in *F. serratus*, mitochondrial markers have been able to detect unique genetic characteristics of the Iberian populations, suggesting these might have been maintained through glacial periods (Hoarau et al., 2007b), contrary to a study considering the same populations based on microsatellites that revealed no unique alleles suggesting these might have been secondarily recolonized following local extinctions (Coyer et al., 2003). On the other hand, the different species within each lineage in *Fucus* cannot be distinguished based on mtDNA (Coyer et al., 2006), only using microsatellites (Billard et al., 2005a), a problem that might be influenced by hybridization creating reticulate inheritance patterns.

Mitochondrial Data

In the last 25 years hundreds of studies have inferred population history using mtDNA (Ballard & Whitlock, 2004; Zink & Barrowclough, 2008). Mitochondria are present in eukaryotic cells, carrying their own DNA (mtDNA) that is usually transmitted maternally, without recombination and with high mutational rate. This way, mtDNA haplotypes, so called because they are inherited as a single non-recombining unit, give us the opportunity to trace genealogical and evolutionary history (Hellberg et al., 2002). However these advantageous features have been associated with animals only, such great level of variation is not found in plants or algae (Zuccarello et al., 1999). Yet, the last decade saw mitochondrial DNA of brown algae species being completely sequenced (Oudot-Le Secq et al., 2001; Oudot-Le Secq et al., 2002; Oudot-Le Secq et al., 2006) and used as a useful molecular marker. *Fucus* phylogeny (Coyer et al., 2006), phylogeography of *F. serratus* (Hoarau et al. 2007b) and *F. vesiculosus* (Hogerdijk, 2008), dispersal history of an invasive kelp (Voisin et al., 2005), were all examined using mtDNA presenting high polymorphism and revealing novel patterns in the evolutionary history of algae populations.

Despite mtDNA being the number one choice for phylogeography studies there is some discussion as to whether it is reliable to use mitochondria as a single marker (Ballard & Whitlock, 2004). A considerable number of studies facing doubts brought nuclear DNA (nDNA) to the picture and in many cases nDNA data supports mtDNA results (Zink & Barrowclough, 2008).

Nuclear Microsatellites

There are several nuclear markers used in population genetics studies but microsatellites (msats) are currently the most popular due to their higher resolution within species. Microsatellites are tandem repeats of a small number of nucleotide (2-10 bp) motifs for which allelic variation is analyzed as size polymorphisms (Hellberg et al., 2002). Msats have been greatly used in *Fucus* and many polymorphic loci have been isolated (Coyer et al., 2003; Engel et al., 2003; Wallace, 2004; Perrin et al., 2007; Coyer et al., 2008). These markers were good predictors in distinguish between *Fucus* taxa (Bergstrom et al., 2005; Engel et al., 2005) and even within a taxon msat could discriminate between populations from distinct habitats (Billard et al., 2005a) and from sympatric and allopatric sites (Engel et al., 2005), as well as between distinctive reproductive strategies in marginal populations (Tatarenkov et al., 2005). Nuclear msat markers have been used to infer hybridization between fucoid species (Coyer et al., 2002; Wallace et al., 2004; Engel et al., 2005; Billard et al., 2005a) and for evidence of polyploidy (Coyer et al., 2006). In a general way, most population genetics studies of this genus have used mainly msat information (Coyer et al., 2003, Coleman & Brawley, 2005; Engel et al., 2005; Tatarenkov et al., 2005; Coyer et al., 2006; Perrin et al., 2007; Tatarenkov et al., 2007). For phylogeography studies, however, given the longer time-scale of the processes that might be involved, nuclear microsatellites are more prone to homoplasy and it is thus better to complement these with other types of variable markers, such as mtDNA.

Objectives

In this study, both mitochondrial and nuclear markers were used to infer the phylogeographic history of *F. spiralis*, including recognition of potential glacial refugia and the subsequent recolonization pathways, and the role of hybridization with *F. vesiculosus* in its phylogeographic patterns. The study also aimed to verify the accuracy of mtDNA in distinguishing the two previously described *F. spiralis* entities.

MATERIAL & METHODS

All laboratory protocols used in this project are described in detail in the Appendix.

Sampling

A total of 733 individuals were collected from 21 locations covering the distributional range of *F. spiralis* (Table I, Fig. 1). Our sample set is composed of individuals collected with different sampling methods, as this was a shared project among different laboratories that collected samples for earlier projects.

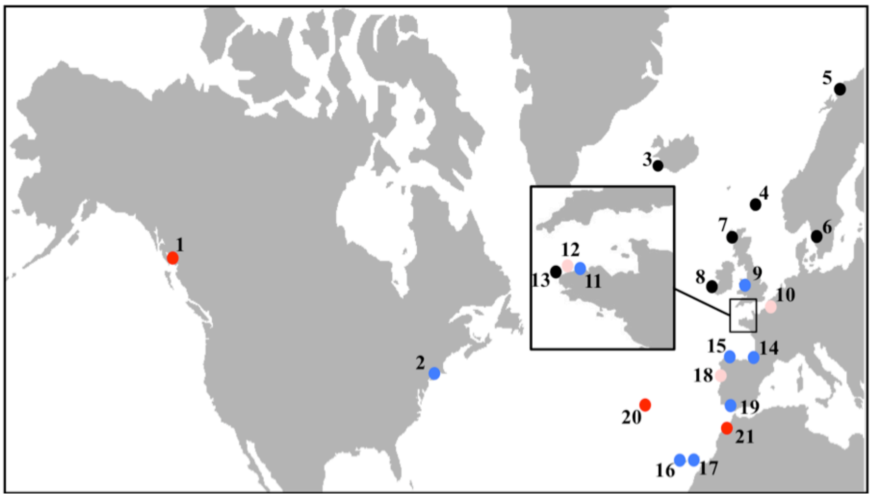

Fig. 1. 21 populations of *F. spiralis* were collected with four sampling methods: Black, a) from Coyer et al., 2006 and Coyer unpublished; Blue, b) this study; Red, c) from Engel et al., 2005 and Perrin et al., in prep; Pink, d) from Billard, 2007. For further sampling details see methods.

Some of the samples were available from other projects prior to this study, thus several sampling methods were used:

a) a 50 m transect along a random walk parallel to the shore at the height where the species is not mixed with *F. vesiculosus* (High shore), where individuals were collected a meter apart from each other (samples from Coyer et al., 2006 and Coyer, unpublished);

b) same as the previous method but, if *F. vesiculosus* was present, instead of one transect, two parallel transects were conducted, one on the upper (High, same as parapatric) and the other on the lower (Low, same as sympatric) distribution range of *F. spiralis*, where it overlaps with *F. vesiculosus* (samples specific for this study);

c) two quadrats 1x1 m distant by ca. 100-300 m (depending on local distributional patchiness) were set in a parapatric zone (i.e., with *F. spiralis* only) and a sympatric zone (i.e., a mixed site with both *F. spiralis* and *F. vesiculosus*), or simply along the *F. spiralis* fringe of highest abundance in allopatry (i.e. if *F. vesiculosus* was absent at the site) to collect at least 24 individuals from each (samples from Engel et al., 2005 and from Perrin et al., in prep).

d) a transect perpendicular/vertical to the shore, sampling 2 *Fucus spp.* individuals at each meter or samples taken using random coordinates within an area of 10 x 2 m, both without *a priori* definition of what the species were to avoid subjective sampling biases (samples from Billard, 2007). For this study only *F. spiralis* individuals were used.

Table I. Sampling locations of *F. spiralis* and sampling method (S) used, as described in the text. **N**, number of individuals per sampling location; **Shore Height,** intertidal sampling positions (Low means overlapping with *F. vesiculosus*); **ND**, shore height was not determined but did not overlap with *F. vesiculosus*; **No FV**, means no *F. vesiculosus* was present; **Unknown**, samples used were from both parapatric (Low) and Sympatric (High) stations, but were mixed; **?**, unknown exact location.

ID	Location	Latitude, Longitude	N	Shore Height	S	Samples from
1/W	San Juan Island, Washington, USA	42º28’N, 123º14’W	48	No Fv	c)	Perrin et al. in prep
2/Ma	Appledore, Maine, USA	42°59'N, 70°37'W	24	ND	b)	this study
3/Ic	Grindavik, Iceland	63°49'N, 22°26'W	24	ND	a)	Coyer et al. 2006
4/Sh	Hamma Voe, Shetlands	60°30'N, 01°34'W	24	ND	a)	Coyer unpub.
5/No	Tromsø, Norway	69°47'N, 19°26'E	24	ND	a)	Coyer unpub.
6/Sw	Goteborg, Sweeden	57°39'N, 11°47'E	24	ND	a)	Coyer unpub.
7/Sc	Oban, Scotland	56°26'N, 05°27'W	24	ND	a)	Coyer et al. 2006
8/Ir	Labaseehda, Ireland	52°36'N,0 9°13'W	24	ND	a)	Coyer unpub.
9/Nb	New Brighton, Liverpool, England	53°26'N, 03°03'W	24 24	Low High	b)	this study
10/Gn	Cap Gris Nez, France	50°52' N, 01°34' W	24	Unknown	d)	Billard, 2007
11/Pl	Port Lazo, Brittany, France	48°45'N, 02°56'W	24 24	Low High	b)	this study
12/Sa	Santec, Brittany, France	48°40' N, 04° 02' W	24 19	Low High	d)	Billard 2007
13/Pe	Perharidy, Brittany, France	48°42'N, 04° 0'W	24	ND	a)	Coyer unpub
14/St	Santoña, Cantabria, Spain	43°26'N, 03°28'W	24 24	Low High	b)	this study
15/Ct	Castello, Asturias, Spain	43°33'N, 06°48'W	24	No Fv	b)	this study
16/Tn	El Medano, Tenerife, Canary Isl	28° 2'N, 16°32'W	24	No Fv	b)	this study
17/Gc	El Burrero, Gran Canaria, Canary Isl	27°54', N 15°23'W	24	No Fv	b)	this study
18/Vc	Viana do Castelo, Portugal	41°41' N, 08°51' W	24 24	Low High	d)	Billard 2007
19/Alb	Albufeira, Portugal	37° 5'N, 08°12'W	24	No Fv	b)	this study
20/Az	Azores, Portugal	?	24	No Fv	c)	Perrin et al. in prep

The sites corresponding to the samples used in this study and that had been taken with each of these methods are represented in Fig. 1. A haphazard subset of 24 individuals from the sample from each site were analyzed, with the exception of samples collected using method c) for which we considered all 48 individuals. After collection samples were stored in silica crystals to achieve a rapid dehydration until DNA extraction.

DNA Extraction

DNA was isolated from 5-10 mg of dried tissue either with NucleoSpin® 96 Plant kit (Macherey-Nagel), DNeasy™ 96 Plant kit (QIAGEN) or CTAB method as described in Hoarau et al. (2007a) but a silica filter plate (Milipore MultiScreen HTS, FB Cat. # MSFBN6B10) was used instead of the silica fines step. For PCR reactions samples extracted with both kits were diluted 1:100 and CTAB extractions 1:10.

Mitochondrial DNA

For mitochondrial haplotyping, the intergenic spacer between 23S ribosomal RNA and tRNA Lys genes (Coyer et al*.,* 2006) was amplified by PCR and analysed either by Single Strand Conformation Polymorphism (SSCP) or by direct sequencing.

Polymerase Chain Reaction (PCR) and Single Strand Conformation Polymorphism (SSCP)

Single-strand conformation polymorphism is a rapid and inexpensive alternative technique to sequencing when the goal is to detect mutations in many samples. SSCP is based on sequence-specific differences in secondary structure of single-strand DNA fragments, resulting in different mobilities when run at low temperature (18ºC-30ºC) under non-denaturing conditions (Orita et al., 1989; Larsen et al., 2007).

The most polymorphic region (*c.* 210bp) of the intergenic spacer (600-700bp) (Coyer et al., 2006) was amplified with the primers FvSSCPf (5'-CCCGTACTAATCCCCATCAGAAGTA-3') and FvSSCPr (5'-GGCTTCTTGATGATTAAAGTCTCAT-3') labelled with FAM and HEX, respectively. PCR reactions were performed in a 10 µL volume containing 1 uL of diluted DNA (1:100 kit extractions, 1:10 CTAB extractions), 1x HotMaster Buffer with 1.5 mM $MgCl_2$ (5Prime), 0.2 mM of each dNTP, 0.15 µM of each labeled primer and 0.045 U 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 as follows: initial denaturation step of 2 min at 94ºC, then 40 cycles of 94ºC for 10 sec, 63ºC for 5 sec, 65ºC for 40 sec, and a final extension at 65ºC for 10 min.

PCR products were diluted 1:200 after cleaning with Sephadex® G-50 (Sigma-Aldrich), and then denatured. SSCP were run in non-denaturing conditions using GeneScan®-350 [ROX]™ Size Standard (Applied Biosystems) on a ABI 3130xl Sequencer (Applied Biosystems). SSCP gels were read using GeneMapper® Software Version 3.7 (Applied Biossytems) and a sample was sequenced for all the different haplotypes, i.e. different peak sizes derived from different electrophoresis mobilities..

Sequencing

The same PCR reaction was performed for sequencing the different haplotypes, but a double volume reaction was prepared with non-labeled primers. PCR products were checked on an agarose gel and a 96-well plate with 10-20ng/µL of each sample was prepared and dried at medium heat in speedvac system. Dried PCR products were cleaned with ExoSAP-IT® (USB Corporation) enzyme. Both strands were cycled-sequenced with reactions consisting of 1µL of BDT-mix, 1µL of primer (5uM), 5 µL Sequence Reaction Buffer and 6 µL of MiliQ water. The sequencing program was conducted on a Veriti™ Thermal Cycler (Applied Biosystems) carried out for 35 cycles of: 15 sec at 96ºC, 50ºC for 1 sec, 60ºC for 4 min with a special feature of each temperature being reached by a soft ramp of 0.3ºC/sec. Samples were once again cleaned with Sephadex® G-50 (Sigma-Aldrich) and dried at medium heat in a speed-vac system. After denaturation in formamide, samples were run on a ABI 3730 Sequencer (Applied Biosystems) and sequences were visualized using Variant Reporter™ Software v1.0 (Applied Biosystems). Sequences were aligned using Bioedit version 7.0.1.

Nuclear DNA: Microsatellites

Billard (2007) has described two genetic entities of *F. spiralis* based on microsatellite loci, two of which were diagnostic, as could also be seen in the analyses of Engel et al. (2005). A total of 91 individuals of *F. spiralis* collected as Low, High and *F. vesiculosus** from Santec and *F. spiralis* from Perharidy were amplified at twelve EST-SSR derived loci (Coyer et al., 2008) and at one of the diagnostic microsatellite loci previously identified: L20 (Engel et al., 2003). EST (Expressed Sequence Tag)-derived SSRs are microsatellites, also called simple sequence repeats (SSR), located within the non-coding untranslated regions (UTRs) of ESTs. ESTs are derived from transcripts (mRNA), and are used to identify and map the position of genes. Therefore, EST-derived SSRs “hitchike” in close association with coding regions, and are frequently used as identifiers of, for example, genes under selection (Eujayl et al., 2002; Li & Brawley, 2004; Coyer et al., 2008). All PCR reactions consisted of a total volume of 10 μL containing 1x HotMaster *Taq* polymerase buffer (5Prime) with 2.5 mM of $MgCl_2$, 1mM of each dNTP, 0.2 μM of each primer, 0.05U HotMaster *Taq* Polymerase and 1μL of diluted DNA 1:10 or 1:100. 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 94ºC for 2 min; 35 cycles of 94ºC for 20 sec, annealing temperature (T_a, Table II) for 10 sec, 65ºC for 35 sec; and a final extension at 65ºC for 10 min.

Table II. Characteristics of the 12 microsatellite loci amplified in 91 *F. spiralis* and *F. vesiculosus* individuals from Santec and Perharidy. Subset of 5 msat loci amplified in 7 other *F. spiralis* populations indicated by +. T_a – Annealing temperature.

Locus	Repeat Array	Primers (5’-3’)	T_a (ºC)	Fluorophore
F-9	$(GT)_{16}$	F: GGCGGAAGTCGATTTGAATA R: ACTTGGCTGACGTCCAGAAT	55	FAM
F-12	$(GT)_{15}$	F: TATGTGTCCGACGACCTGAG R: TGAAGTCAAATGCTTGTTCG	55	FAM
F-17	$(AT)_7$	F: GCAGACAGAGAGGGCAGAAG R: CCCCTCTCTCCCAGGTATTT	55	HEX
F-21$^+$	$(TG)_{15}$	F: CATGTAGCGTGAAGCGTTTG R: CACGCAAACAAAACGTCAAC	55	FAM
F-34$^+$	$(TG)_6$	F: TGCCGAAGTACCGCATCTAC R: CTCCACTGGCATGCTGTTTA	55	FAM
F-36$^+$	$(TG)_8$	F: TTTGCGGGATTGAAAGAGAG R: CCAGAATGGATGGGAAGAAA	55	HEX
F-42$^+$	$(AGC)_5$	F: AGTGTGACTGCCCATTAGGG R: AGACGTAACCCAGTGCTGCT	55	FAM
F-49$^+$	$(AT)_6$	F: TGCTGTAGAAGGCCGAAGTT R: AACGAGTTCGTCGAGTGTCC	55	HEX
F-58	$(TA)_8$	F: CGTGTTTTGTCCGTCCTTTT R: CGGAACAGATGGGAGACAAT	55	FAM
F-59	$(AT)_7$	F: TCGCCATATCTGTGTCAAGG R: AACAAATTGGTGCCGAGTGT	55	HEX
F-60	$(CA)_8$	F: GGGGTTGTTTTCGATAAAAGG R: GCAATCGACCTCGAGAAATC	55	FAM
F-72	$(AG)_6$	F: ATCTCCGCCTTAACCCAGTC R: CAGCTGGATACGGATGGAGT	53	FAM
L20	$CTGG(CTG)_8(TTG)_3CTT$	F-ACTCCATGCTGCGAGACTTC R-CCTCGGTGATCAGCAATCAT	52	FAM

A subset of five EST derived microsatellites were amplified at 264 *F. spiralis*

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* *F. vesiculosus* individuals belong to another sample set. They were included in order to infer weather we were able to distinguish *F. spiralis* and *F. vesiculosus.*

individuals from seven other locations: New Brighton (NW England), Port Lazo (Brittany, France) Santoña (Cantabria, North Spain), Viana do Castelo (North Portugal), Albufeira (South Portugal), Oualidia (Morocco) and Tenerife (Canary Island, Spain). The same conditions and program as described above were used. PCR products were run on a ABI 3730 Sequencer (Applied Biosystems) and visualized with GeneMapper® Software Version 3.7 (Applied Biossytems).

Data Analysis

Mitochondrial DNA

Relationships among mt-IGS haplotypes were inferred using TCS vs. 1.21 (Clement et al., 2000). TCS creates a network with the most parsimonious branch connections between sequences with 95% confidence and estimates the haplotype outgroup probabilities correlated with their age, allowing the identification of the ancient haplotype. Haplotypic (h) and nucleotide (p) diversities were calculated with Arlequin software vs. 3 (Excoffier et al., 2005).

Nuclear DNA

Multilocus genotypes were analyzed using the software Structure version 2.2 (Pritchard et al., 2000). Two sets of multilocus data were considered in this analysis: the 91 individuals amplified at 12 microsatellite loci in both *F. spiralis* and *F. vesuclosus* individuals; and the subset of 5 msat amplified in 264 *F. spiralis* individuals. Structure uses a Bayesian clustering approach to assign individuals to populations based on their genotypes. Basically it assumes a K number of populations that are characterized by allele frequencies at each locus and cluster the individuals to each population based on their multilocus genotypes. As noted by Perrin (in prep) and in our mtDNA results four different entities were expected in the 12 msat set (*F. spiralis* South, *F. spiralis* Low, *F. spiralis* High and *F. vesiculosus*) therefore a K=4 of parental populations were assumed. For the 5 msat subset the goal was to differentiate the three *F. spiralis* entities so a K=3 was defined. For both data sets analyzed a burn-in period of 2 x 10^5 repetitions and a run length of 10^6 steps were used. The 'burn-in' period corresponds to the time that the simulation should run before it starts collecting data to minimize the effect of the random starting configuration. Afterwards the simulation should run long enough to achieve accurate parameter estimates and this is defined by the 'run length' (Pritchard et al., 2000). For the 12 msat dataset, which includes *F. spiralis* collected as High and Low and *F. vesiculosus*, a factorial correspondence analysis (FCA) was carried out using Genetix software version 4.03 (Belkhir et al., 2001). FCA plots each individual genotype in the dataset as a function of axes derived to explain the largest proportion of the total variability in the data set. Individuals with three or more missing data were excluded of the analysis. Allelic frequencies and richness (*A*), the last standardized to n=4, were calculated with R software using StandArich v.1.00 package (Alberto, 2006). Expected heterozygosity (H_{exp}) was calculated with Genetix 4.03 (Belkhir et al., 2001). These parameters were calculated by entity as they were determined by the multilocus genotypes.

RESULTS

Notation used:

Coll. represents the shore height where the sample was collected.

> ***F. spiralis*** **coll. High/Low** = *F. spiralis* collected on the high/low shore (where low shore corresponds to the zone where the species is mixed with *F. vesiculosus*).

Det. represents what cluster the multilocus microsatellite genotype was assigned to.

> ***F. spiralis*** **det. High/Low** = *F. spiralis* determined by genotype assignment to belong to the genetic cluster named *F.spiralis* High/Low.

Mitochondrial DNA

A total of 16 haplotypes (h1-h16) were found in the 210 bp mitochondrial intergenic spacer in 733 *Fucus spiralis* individuals (Fig.2).

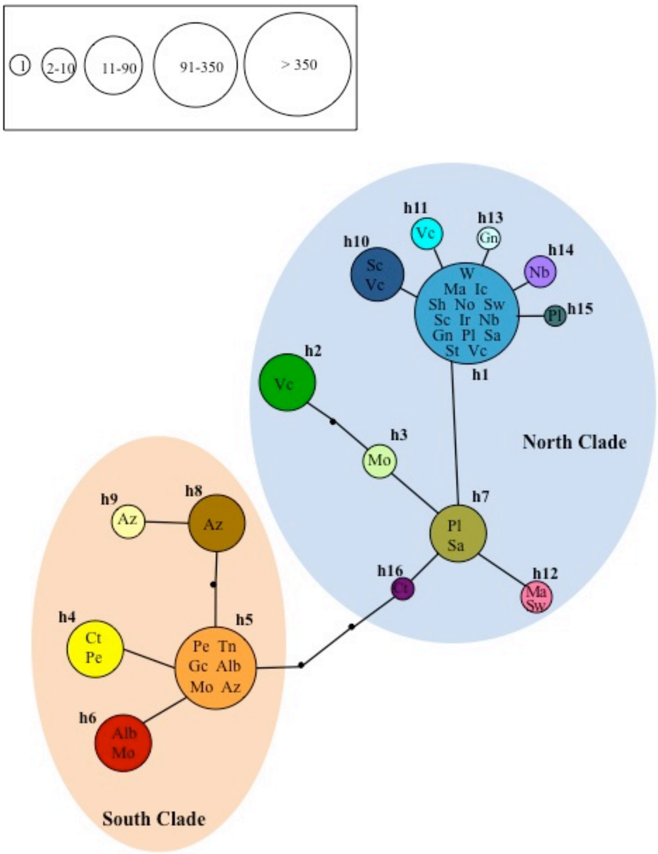

Fig. 2. Statistical parsimony network of *F. spiralis* mtIGS haplotypes. Each circle represents one haplope and letters the locations where they were found (as Table I and III). Circle size is proportional to the number of individuals with the given haplotype as shown in legend above. Each branch between two nodes represents one mutational step and small black nodes represent haplotypes with intermediate mutation steps not found in our samples.

Haplotype 1 (h1) was found in 52% of the samples and was the most widely distributed one from Viana do Castelo to Norway, Iceland and the United States on both Atlantic and Pacific coasts. The second most common haplotype, h5, was present in 18% of the samples but only in southwestern Atlantic populations: Albufeira, Azores, Morocco, Canary Islands and 1 individual in Perharidy (Brittany). We named h5 and their closely related haplotypes (h4, h6, h8 and h9) South Clade and h1 and all the other haplotypes North Clade, to represent their main geographic occurrence (Fig.2, Fig.3).

The two most common haplotypes, h1 and h5, never occurred together in the same population and the haplotypes that were present together with them in a given population were always closely related with the exception of Oualidia, Morocco (21 in Fig. 3) and Castello, North Spain (15 in Fig. 3). These populations contained both South and North Clade haplotypes. In Morocco, out of 48 individuals, 4 had an endemic North Clade haplotype (h3) and in Castello one individual had a North Clade haplotype (h16) while all the other individuals in the sample had South Clade ones. Castello and Perharidy in Brittany (15 and 13 in Fig. 3, respectively) were the only two populations north of Viana do Castelo containing haplotypes from the South Clade.

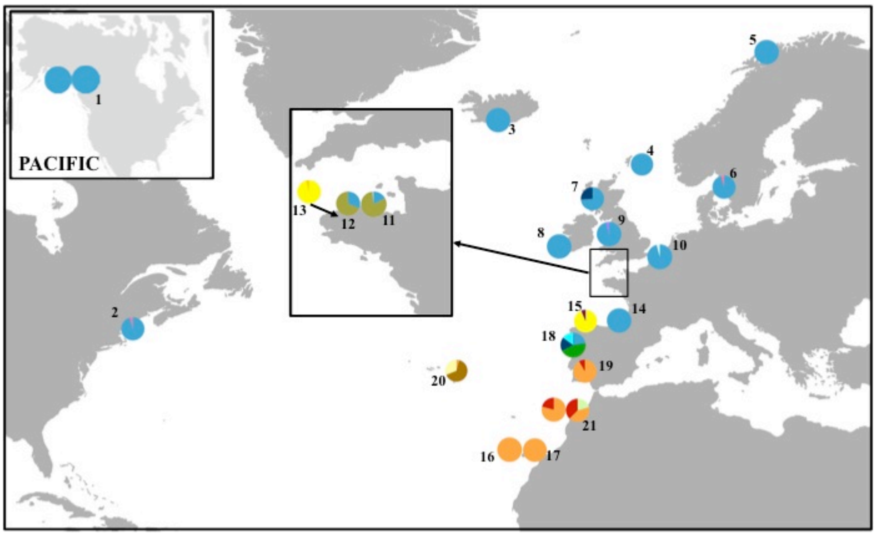

Fig. 3. *F. spiralis* mt-IGS haplotype distribution. Each color represents a different haplotype from h1 to h16 as Fig 2. Numbers in the map correspond to population ID on Tables I and III.

Nuclear Data: Microsatellites

Testing Novel msats for Distinction of F. spiralis *entities*

We analyzed a total of 91 individuals of *F. spiralis* and *F. vesiculosus* from locations in Brittany at 12 microsatellite loci in order to assess the ability of newly developed msat loci (Coyer et al., 2008) to resolve relationships between these species and between the *F. spiralis* entities that had been described using the microsatellite loci of Engel et al. (2003) and Perrin et al. (2007). With the recent discovery of two *F. spiralis* genetic entities in the North Clade (Billard, 2007), the samples to be here analysed were chosen to be the same individuals identified by Billard (2007) as *F. spiralis* High and Low, in Santec, Brittany, and a second sample of *F. spiralis* from Perharidy that is geographically very close to Santec and the only population north of Iberia in which we found South Clade haplotypes.

Multi-locus genotype clustering assignment was achieved using the software Structure (Pritchard et al., 2000) considering four parental groups (K=4), based on the previous knowledge (Billard, 2007; Perrin et al., in prep.) that two or three clusters occur in *F. spiralis*. Four clusters were thus defined: three of *F. spiralis* and one of *F. vesiculosus*, with few intermediates between these entities (Fig. 4). The clusters *F. spiralis* det. Low and det. High were both found in Santec, where they share the intertidal zone with *F. vesiculosus*. A third entity was found in Perharidy, named *F. spiralis* South (because it is genetically similar to the South cluster individuals, see below).

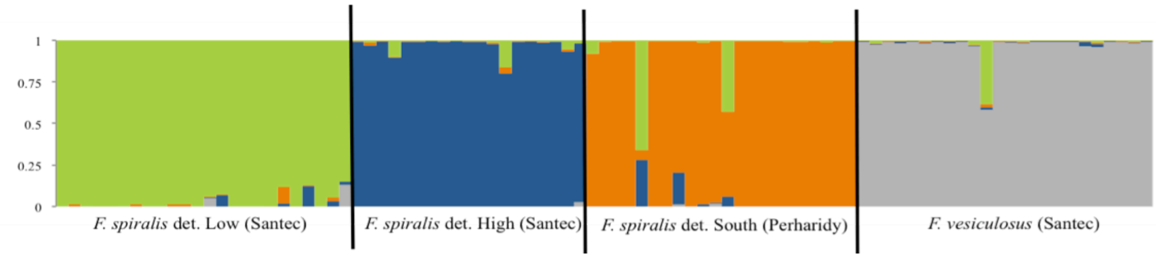

Fig. 4. Bayesian analysis with the Structure software based on 12 msat loci for populations of *F. spiralis* and *F. vesiculosus* from Santec and *F. spiralis* from Perharidy. Each vertical bar is the multi-locus genotype of one individual. Four different entities were identified: Green, *F. spiralis* det. Low (Santec); Blue, *F. spiralis* det. High (Santec); Orange, *F. spiralis* det. South (Perharidy); Grey, *F. vesiculosus* (Santec). (K=4; MCMC=1000000).

The genetic distance between these four entities can be visualized using a factorial correspondence analysis (FCA), corroborating the existence of three *F.*

spiralis clusters. The first axis explains approximately 20% of the distance between individuals (Fig. 5), separating *F. vesiculosus* from all *F. spiralis*, and revealing much more divergence between *F. vesiculosus* individuals than between *F. spiralis*, where the southern cluster appears to be the most divergent from *F. vesiculosus*.

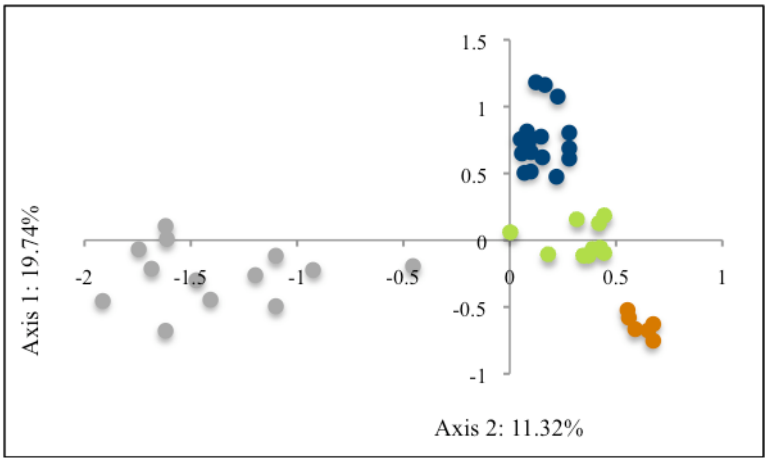

Fig. 5. Factorial correspondence analysis (FCA) based on 12 msat of *F. spiralis* and *F.vesiculosus* individuals from Santec and *F. spiralis* from Perharidy. Each point represents one individual. Green, *F. spiralis* det. Low (Santec); Blue, *F. spiralis* det. High (Santec); Orange, *F. spiralis* det. South (Perharidy); Grey, *F. vesiculosus* (Santec).

Diagnostic Power of 12 msat vs 5 msat Subset

As seen before, the 12 microsatellite loci were able to distinguish three *F. spiralis* entities. Based on F_{ST} values and allelic frequencies (Appendix F) of these loci we chose the 5 loci that better differentiate the three *F. spiralis* entities. We analyzed the same samples of *F. spiralis* from Santec and Perharidy with the 5 msat subset using Structure and the same three clusters were defined. The 5 msat loci analysis shows more intermediate genotypes (and one totally assigned to a different group) than using the 12 loci (Fig. 6). However we can interpret this as lower resolution caused by the smaller number of loci, rather than as revealing more hybridization. Nevertheless, the three *F. spiralis* entities were well defined with either number of microsatellites.

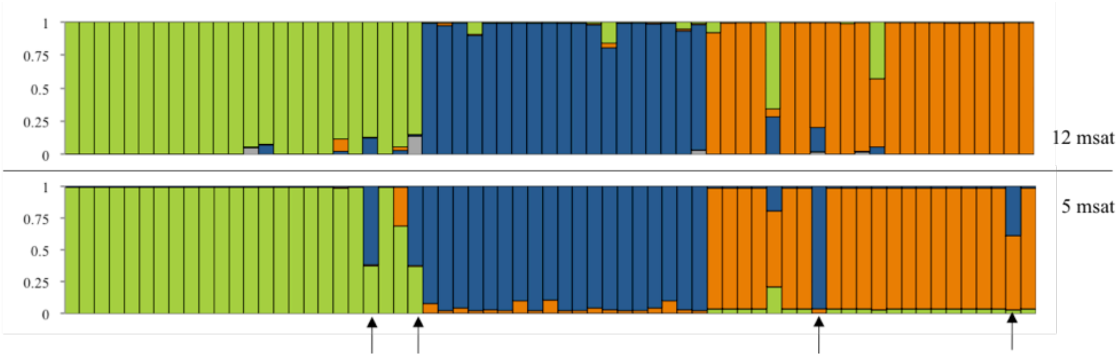

Fig. 6. Comparison of bayesian analyses with Structure software based on 12 and 5 microsatellite loci for *F. spiralis* populations: Green, Santec *F. spiralis* det. Low; Blue, Santec *F. spiralis* det. High; Orange, Perharidy *F. spiralis* det. South. Each vertical bar represents one individual that is the same in the upper and lower panel. The 5 msat is a subset of the 12 msat original set. The colour bars indicate the proportion of the genome assigned to each cluster. Arrows indicate individuals with variations in genome assignment when the subset is used (K=3; MCMC=1000000).

These comparisons revealed that these 5 msats were in most cases sufficient for general assignment of populations to specific clusters. Although a few individuals (6%) may lose accuracy in the assignment, this is not considered significant for a phylogeographic study, thus it was considered that genotyping the extra 7 msats was not worth the cost in relation to the benefit. Using either of these new combinations of 12 or 5 msats it was possible to identify the same clusters as had been identified using the 9 msats from Engel et al. (2003) and Perrin et al (2007) together.

Nuclei and Mitochondria

Combination of Data from Both Genomes

When the nuclear microsatellites and the mitochondrial data sets are combined for the samples previously analysed with 12 msats (see Fig. 7, but it illustrates the combined data using the 5 msat data in order to allow for comparisons with the other samples that were analysed with only 5 msat loci), the three *F. spiralis* entities that had been recovered with nuclear microsatellites had distinct mitochondrial haplotypes associated with each. These corresponding haplotypes were, in Santec, Brittany: h7 in *F. spiralis* Low individuals and h1 in *F. spiralis* High (Fig. 7). The third entity identified in Perharidy by the msat data as *F. spiralis* South (Fig. 4), had the corresponding mitochondrial haplotypes also belonging to the Southern Clade.

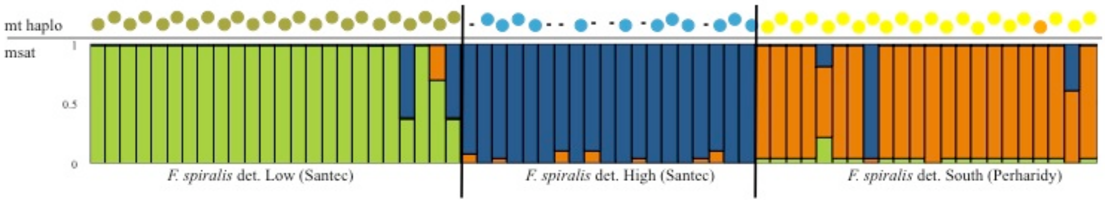

Fig. 7. *F. spiralis* mitochondrial haplotypes (mt haplo) aligned with Structure genotype assignment analyses based on 5 msat loci taken from Fig. 6 (msat). For mt haplotypes, each circle colour represents a different haplotype (colour codes as in the network, Fig. 2); mitochondrial missing data are represented with a dash (-). Msat legend is the same as Fig 6.

In order to better describe the correspondence between microsatellite-defined *F. spiralis* clusters and their mt haplotypes, a total of 264 *F. spiralis* individuals from 7 other locations (chosen to be representative of the three msat-determined clusters), were amplified at the subset of 5 msat loci previously shown to be capable of distinguishing High and Low *F. spiralis*. These revealed three entities in these 7 sites: *F. spiralis* Low was found in Port Lazo (Brittany), Viana do Castelo (North Portugal) and Morocco; *F. spiralis* High in Port Lazo, New Brighton (NW England), Santoña (North Spain) and Viana do Castelo; and *F. spiralis* South present in Perharidy was now found in the Southern populations of Albufeira, Tenerife and Morocco (Fig. 8).

Sampling was based on shore height without recording morphology (except for the samples from Billard, 2007). Therefore, despite being collected as "*F. spiralis* Low" or "*F. spiralis* High", these classifications of individuals based on sampling height were in several cases not coincident with the multilocus genotype. In some sites only one *F. spiralis* entity was detected on both the low and high shore, such as in New Brighton (NW England, 9 in Fig. 3) and Santoña (NE Spain, 14 in Fig. 3), where every individual collected on the low shore was actually *F. spiralis* High (Fig. 8b and 8c). In these sites, there was no evidence of *F. spiralis* Low being present except for one individual collected on the high shore in New Brighton, although this is within the expected assignment error rate of 6% found above when comparing with 12 msat assignments. In other cases there are vast areas of overlapping distribution (e.g., in North Portugal, pers. obs.). In Port Lazo, Brittany, (Fig. 8a) several individuals collected as one of the entities appear to be the other one based on at least one of the two genotypes analysed. In Viana do Castelo, North Portugal (18 in Fig. 3), 6 individuals collected as *F. spiralis* Low were determined as *F. spiralis* High (Fig. 8d). Furthermore, some of these 5 loci show private alleles for one of these genetic entities

(Fig. 9). F21 and F34 contain almost 100% of distinct alleles for *F. spiralis* High and *F. spiralis* Low while F36 seems to contain private alleles for the *F. spiralis* South entity, even if present in low frequencies in a few High individuals. In general, there are shared alleles between *F. spiralis* Low and *F. spiralis* South (F21 and F42) and *F. spiralis* High and South (F34 and F36).

There is an agreement between the information from both genotypes in general (97% of the individuals), although this is not always the case. Regardless of the sampling identification, we can in most cases match the msat-determined entities with distinct mt-IGS haplotypes. In *F. spiralis* South, Southern Clade haplotypes were found (Fig. 8e and 8f) and *F. spiralis* Low and High had Northern Clade haplotypes. Furthermore, within the Northern Clade haplotypes, those found in *F. spiralis* determined as Low were different from the ones in *F. spiralis* determined as High (Fig. 8a, 8b, 8c and 8d). The only southern population where an entity other than *F. spiralis* South was found was in Morocco (Fig. 8f), where four individuals were not only determined by the msat as *F. spiralis* Low but the mitochondrial haplotype was also a Northern Clade one.

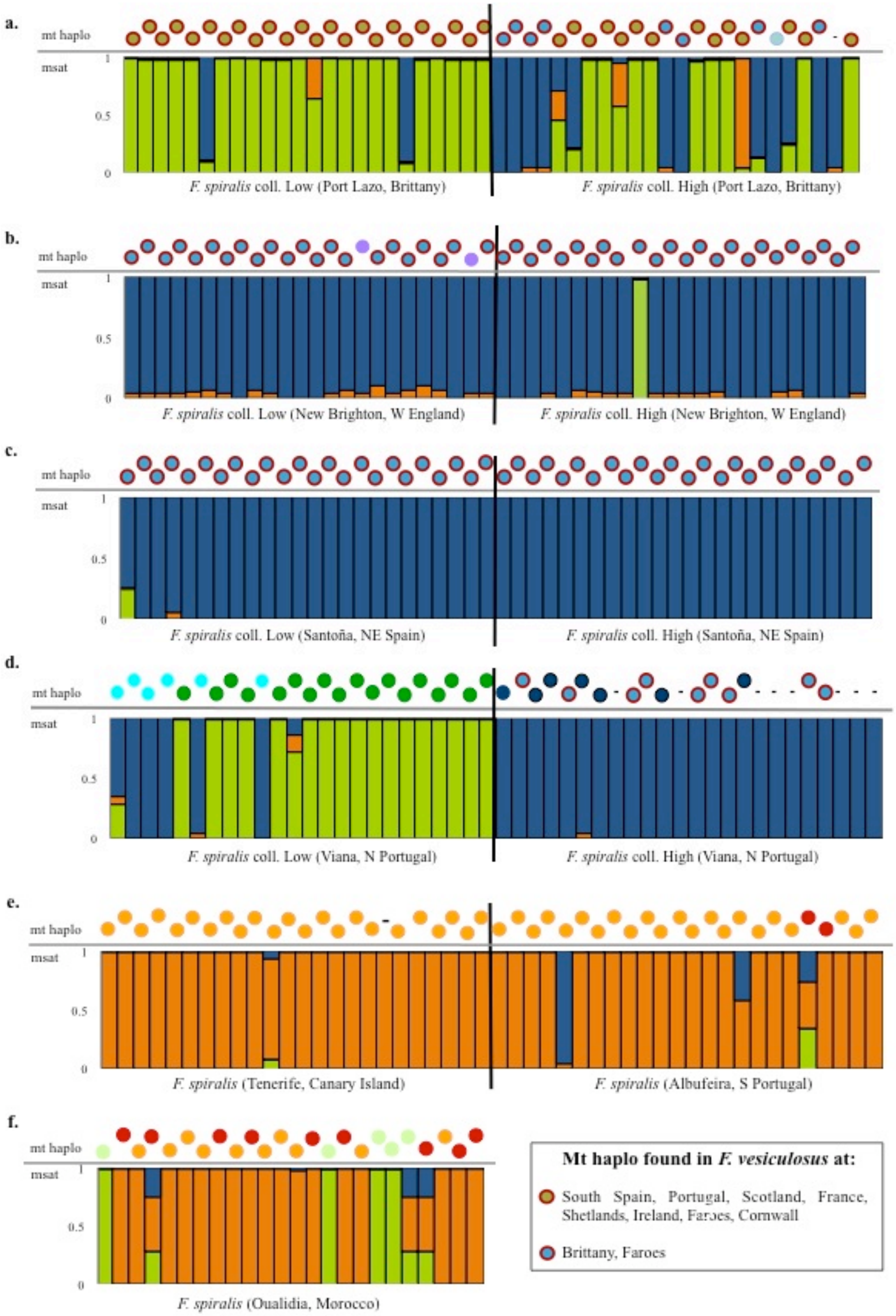

Fig. 8. Proportion of the multilocus genotype of *F. spiralis* assigned to each of three clusters after analyses with Structure based on 5 msat loci (msat) and corresponding mitochondrial haplotype (mt haplo). Each vertical bar represents the multilocus genotype of one individual with its mt genotype shown in the circle above. Circle colours represent distinct haplotypes as Fig. 2. Green, *F. spiralis* det. Low; Blue, *F. spiralis* det. High; Orange, *F. spiralis* det. South. The red line circle indicates that those haplotypes were also found in *F. vesiculosus*. Individuals are divided and labeled according to their position on shore (*F. spiralis* coll. Low/coll. High). (K=3; M=1000000).

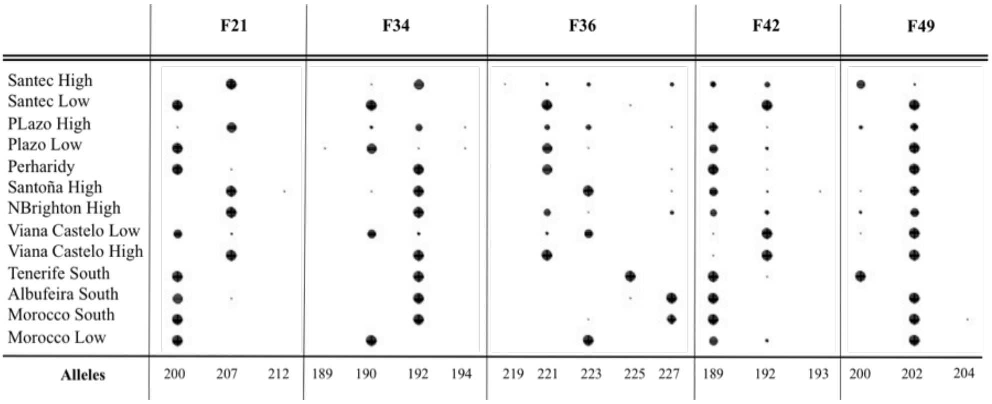

Fig. 9. Allelic frequencies of the 5 msat loci subset analyzed in the 7 populations of *F. spiralis* represented as multilocus assignment determination (*F. spiralis* Low, High or South). The circle indicates the presence of the allele and its diameter is proportional to the frequency of that allele present in the sample.

A study conducted at the same time as this one used the same mitochondrial intergenic spacer for *F. vesiculosus* (Hogerdijk, 2008). Given the known hybridization of this species with its closely related congener *F. spiralis* we compare that information here to try to infer their relationship and any sign of introgression based on their mt-IGS haplotype networks (Fig. 10). In agreement with the previously found lack of resolution of this marker between these two species (Coyer et al., 2006), this analysis did not reveal these species as separate networks, not even a network with only eventual rare events of haplotype sharing, as might be expected by rare, non selected for, events of hybridization. Rather, the resulting network clustered *F. spiralis* North Clade haplotypes with *F. vesiculosus*, with three shared haplotypes between the two species. Haplotype 7 (h7), frequently found in both *F. vesiculosus* and *F. spiralis* Low, was suggested by TCS as the shared ancestral haplotype. The network is very shallow, with very short branch lengths (i.e., few mutational steps along the branches), with more steps within species than between the two distinct species, revealing recent divergence between all haplotypes here found and no distinction of haplotypes between species that are otherwise clearly distinct based on msats (Billard et al., 2005a). The sites where *F. spiralis* was shown to have mt haplotypes that can also be found in *F. vesiculosus* are all located to the North of Viana do Castelo (Fig. 8), the northern boundary of allopatric populations where the two species do not occur together (Ladah et al., 2003). Yet, the shared haplotype with *F. spiralis* was in general found in *F. vesiculosus* not only from the same site but widely from South Spain (Ayamonte) to the Shetlands (Fig. 8).

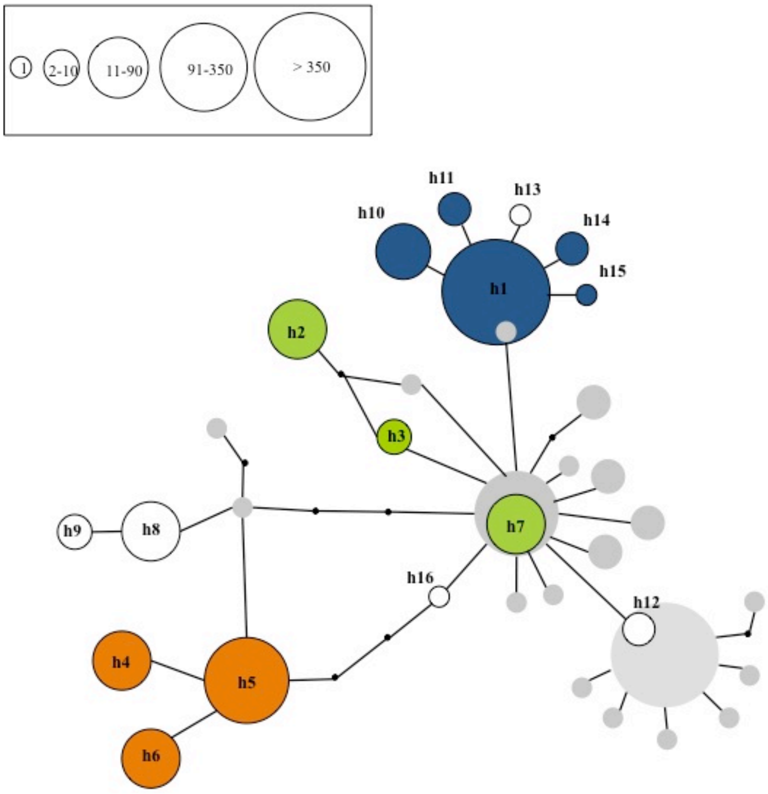

Fig. 10. *F. spiralis* and *F. vesiculosus* mt haplotype network. Coloured haplotypes (h1-h16) were found in *F. spirali*s, *F. vesiculosus* haplotypes are coloured grey and are not numbered. The size of each circle is proportional to the number of individuals presenting that haplotype (see legend above) and each branch represents one mutation step. Haplotypes shown overlapped are those found in both species. Colour describes microsatellite determination: Orange, haplotypes found in individuals determined as *F. spiralis* South; Green, haplotypes present in individuals determined as being *F. spiralis* Low; Blue, *F. spiralis* High haplotypes; Blank, unknown multilocus genotype (not determined).

Distribution of F. spiralis *clusters*

Considering now that three different *F. spiralis* clusters are distinguishable by combining microsatellites and mitochondrial data, we found it pertinent to redraw the haplotype distribution map considering this separation into three main types rather than the two major ones initially revealed by the mitochondrial data alone (Fig. 11). Geographically, the msat-determined *F. spiralis* Low and High both represent Northern Clade haplotypes in all populations North of Viana do Castelo. *F. spiralis* determined as South with both msats and mt haplotypes were found in all Southern Populations, but also in North Iberia (Castello) and Brittany (Perharidy). Most of the distributional range of the species, the Northern areas throughout the North Atlantic and the Pacific, were mainly composed of a single type, the Northern High cluster, with very low, if any, divergence between sites. The South is mainly comprised by southern cluster haplotypes, and the most diverse distributional region for this species, where all various types of haplotypes are present, is found in the south-middle region that corresponds mostly to the Iberian Peninsula and Brittany.

It is clear from the analysis of Fig. 11 that most of the diversity of the species is not present within particular populations but rather divided into low diversity clusters that are very distinct among them. The previous apparent higher haplotypic diversity in some populations was at least in part due to mixing haplotypes found in different entities in the same site. In particular for Viana do Castelo, where the occurrence of 4 haplotypes appears as the highest haplotypic diversity found throughout the range (Fig. 3), this is partly due to the joint occurrence of two differentiated genetic clusters (Fig. 11). An identical phenomenon is observed in Brittany and Morocco. Thus, in order to understand temporal patterns of diversity changes, as might have been caused by glacial periods, it is thus of interest to perform diversity calculations keeping these entities separate.

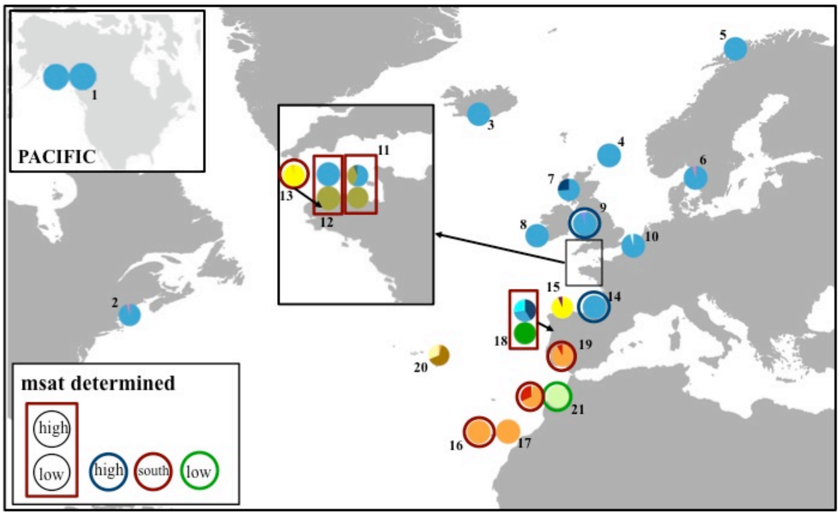

Fig. 11. *F. spiralis* haplotype distribution map considering the microsatellite determined entities as represented in the bottom-left box. Each color represents one distinct haplotype (color code as Fig 2). Numbers are location IDs (Table I and III). Size of the circles is not proportional to the number of individuals with the given haplotype.

Genetic Diversity

Diversity parameters were calculated separately for each of the three msat-determined entities per site. The msat genotyped populations were represented by low polymorphism with allelic richness between 1 and 2 alleles per locus per population and four populations had private alleles: Port Lazo (11 in Fig. 11), Santec (12 in Fig. 11), Santoña (14 in Fig. 11) and Morocco (21 in Fig. 11) (Table III). Expected heterozygosity (H_{exp}) was never higher than that in Port Lazo (0.394), Brittany.

A maximum of 3 mitochondrial haplotypes were found in the same population. Private haplotypes were detected in 8 populations: Morocco, Azores, Viana do Castelo in both *F. spiralis* Low and High, Castello, Port Lazo, Cap Gris Nez and New Brighton. Haplotypic diversity (*h*) ranged from 0 to 0.6883 and the highest nucleotide diversity (*p*) was 0.00414. The highest values for both of these parameters were registered for *F. spiralis* High in Viana do Castelo. Thus, even after calculating diversities separately for each entity, Viana do Castelo continued to have the greatest mitochondrial diversity.

Table III. Sampling locations and diversity measures for nuclear and mitochondrial loci in *F. spiralis*. **ID**, identification as Fig. 1, 2, 3 and11; **N**, number of individuals per sampling location used; **Shore Height,** intertidal sampling positions (Low means overlapping with *F. vesiculosus*); **ND**, shore height was not determined but did not overlap with *F. vesiculosus*; **No FV**, means no *F. vesiculosus* was present; **Unknown**, samples used were from both parapatric (Low) and Sympatric (High) stations, but were mixed. **N'**, number of individuals of each entitie determined by msat, used to all diversity calculations; ***A***, multilocus allelic diversity; $\mathbf{H_{exp}}$, expected heterozygosity; ***h***, haplotype diversity (bold=private); ***p***, nucleotide diversity.

ID	Location	Shore Height	N	Entitie det. based on 5msat loci			N’		*A*	H_{exp}	Private Alleles	mt-IGS spacer		
				Low	High	South						Haplotypes	*h*	*p*
1/W	San Juan, Island, Washington, USA	No Fv	48	-	-	-	-	-	-	-	-	h1	0	0
2/Ma	Appledore, Maine, USA	ND	24	-	-	-	-	-	-	-	-	h1, h12	0.1111	0.00106
3/Ic	Grindavik, Iceland	ND	24	-	-	-	-	-	-	-	-	h1	0	0
4/Sh	Hamma Voe, Shetlands	ND	24	-	-	-	-	-	-	-	-	h1	0	0
5/No	Tromø, Norway	ND	24	-	-	-	-	-	-	-	-	h1	0	0
6/Sw	Goteborg, Sweeden	ND	24	-	-	-	-	-	-	-	-	h1, h12	0.0833	0.00079
7/Sc	Oban, Scotland	ND	24	-	-	-	-	-	-	-	-	h1, h10	0.4032	0.00192
8/Ir	Labaseehda, Ireland	ND	24	-	-	-	-	-	-	-	-	h1	0	0
9/Nb	New Brighton, Liverpool, England	Low	24	0	24	0	Low	0	-	-	-	-	-	-
		High	24	0	24	0	High	48	1.5 ± 02	0.282	0	h1, **h14**	0.0816	0.00039
10/Gn	Cap Gris Nez, France	Unknown	24	-	-	-	-	-	-	-	-	h1, **h13**	0.0833	0.00039
11/Pl	Port Lazo, Brittany, France	Low	24	22	2	0	Low	32	1.4 ± 0.2	0.166	1	h7	0	0
		High	24	10	13	1	High	15	2 ± 0.3	0.394	0	h1, h7, **h15**	0.5824	0.00304
12/Sa	Santec, Brittany, France	Low	24	24	0	0	Low	24	1.1 ± 0.1	0.049	0	h7	0	0
		High	19	0	19	0	High	19	1.8 ± 0.2	0.281	1	h1	0	0
13/Pe	Perharidy, Brittany, France	ND	24	0	0	24	South	24	1.2 ± 0.2	0.065	0	h4, h5	0.0833	0.00039
14/St	Santoña, Cantabria, Spain	Low	24	0	24	0	Low	0	-	-	-	-	-	-
		High	24	0	24	0	High	48	1.3 ± 0.3	0.157	2	h1	0	0
15/Ct	Castello, Asturias, Spain	No Fv	24	-	-	-	-	-	-	-	-	h4, **h16**	0.125	0.00238
16/Tn	El Medano, Tenerife, Canary Isl	No Fv	24	0	0	24	South	24	1 ± 0.1	0.08	0	h5	0	0
17/Gc	El Burrero, Gran Canaria, Canary Isl	No Fv	24	-	-	-	-	-	-	-	-	h5	0	0
18/Vc	Viana do Castelo, Portugal	Low	24	18	6	0	Low	18	1.5 ± 0.3	0.022	0	**h2**	0	0
		High	24	0	24	0	High	30	1 ± 0.1	0.032	0	h1, h10, **h11**	0.6883	0.00414
19/Alb	Albufeira, Portugal	No Fv	24	0	0	24	South	24	1.2 ± 0.2	0.066	0	h5, h6	0.159	0.00076
20/Az	Azores, Portugal	No Fv	24	-	-	-	-	-	-	-	-	h5, **h8**, **h9**	0.502	0.00294
							Low	4	1.2 ± 0.1	0.086	0	**h3**	0	0

DISCUSSION

Species or Not Species

Since Darwin's revolutionary "Origin of Species" (1859), speciation phenomena and species concept have been a major topic and even philosophical theme of inflamed debates. At least a dozen different species concepts have been proposed since then: Biological Species Concept, Cladistic Species Concept, Cohesion Species Concept, Ecological Species Concept, Evolutionary Species Concept, Genetic Species Concept, Genotypic Cluster Concept, Phenetic Species Concept, Phylogenetic Species Concept, Recognition Species Concept and Reproductive Competition Concept (Hey, 2001; Lee, 2003; Mallet, 2007), just to name some. An agreement between taxonomists, evolutionists, ecologists and biologists on this matter seems to have a long road ahead.

The major problem in defining if a species evolves into two different species or not relies on the speciation process. As a gradual process there will be a time during speciation where gene flow is present but restricted and we cannot discern the existence of one or two species (Lee, 2003).

The results of this thesis raise the question: are we witnesses of a speciation process in *F. spiralis*? Our results support the hypothesis of an ongoing speciation process in progress given the observed divergence of distinct entities not only between geographically distinct regions but also in sympatry (though parapatric habitat segregation, see below). Our nuclear and mitochondrial markers in combination recovered three different entities in *F. spiralis*: one mainly present in its South distributional range where the species is allopatric with any congeners, and two in the Northern populations where it is sympatric with its closely related sister species, *F. vesiculosus*.

The occurrence of differentiated populations without significant gene flow within a species is common and expected, but only so among populations that are geographically isolated or separated by some type of dispersal barrier, as maybe the case of *F. spiralis* South and North (see below). The discovery of differentiated entities in sympatry, described in our study, is thus of remarkable interest as no obvious barriers to gene flow are known between *F. spiralis* High and *F. spiralis* Low occurring on the same shore, besides some degree of vertical zonation along the intertidal zone (therefore in a strict way we call these *sensu latu* sympatric types as parapatric entities, given that their habitats contact but do not completely overlap).

The South Clade: F. spiralis *South*

Two clades were identified with our mtDNA intergenic spacer in *F. spiralis*. Given the haplotype geographic distribution range of each clade, we named them South and North Clades.

South Clade haplotypes were strictly found in Southern Populations where *F. spiralis* populations are allopatric to *F. vesiculosus* and in two populations in the North: Castello (North Iberia) and Perharidy (Brittany). The mtDNA haplotypes found in *F. spiralis* South are at least three mutational steps away from the closest haplotype found in other entities of the same species.

In the south, *F. vesiculosus* is not present on open coastal shores due to the reduced recruitment and low survivorship in exposed areas without canopy (Ladah et al., 2003). Consequently, this species is found only in estuaries and coastal lagoons in their lower latitudinal distribution range, where they might be under less desiccation and photoinhibitory stress in those environments (Ladah et al., 2003). Conversely, *F. spiralis* as a high intertidal species, is more adapted to emersion stressors such as high and low temperatures and desiccation (Chapman 1995; Davidson & Pearson, 1996) appearing therefore, more able to survive harsher conditions prevailing in the South. *F. spiralis* has thus been living in the absence of congeneric species in the coastal habitats of these southern latitudinal ranges, providing the opportunity for greater differentiation from its closely related sister species. This differentiation in the mitochondria is also clear in nuclear microsatellites, and these individuals form a distinct cluster of multilocus genotypes.

The well supported Southern entity defines to some extent (i.e., with the exception of a few occurrences detected further north) an accentuated genetic break observed along the Western coast of Iberia: *F. spiralis* South in Southern Portuguese populations and *F. spiralis* Low and High from Viana do Castelo to the north. The occurrence of a North-South biogeographic boundary along the Portuguese coast is a pattern already recognized by different authors in algae (Alberto et al., 1999), seagrasses (Diekmann et al., 2005) and in groundfish assemblages (Gomes et al., 2001).

Several factors may influence the existence of this pattern. Along the Iberian West Coast some strong upwelling systems act to promote water movements perpendicular to, rather than along, the shore (Fiuza, 1983), which can prevent free connectivity between Southern and Northern marine populations. The Tagus River that flows into the Atlantic, and like any other large river affects salinity gradients and near-shore currents, could also potentially act as biogeographical barrier. Distant approximately 130 km from the Tagus River mouth there is the most imposing factor for the North-South biological split: the Nazaré Canyon. This is one of the largest submarine canyons in Europe that extends for 230 km reaching a depth of 5000 m (Koho et al., 2007) and it has been suggested that it is responsible for some changes in current circulation patterns (Gomes et al., 2001; Koho, et al. 2007). Despite the occurrence of these putative breaks, the nature of this North-South biogeographic distinction along the Iberian Peninsula is still poorly understood. *F. spiralis* gamete dispersal is however likely to be extremely restricted, as *Fucus* gametes in general are (e.g., Serrao et al., 1996; Ladah et al. 2008), thus most likely to be influenced by surface wind-driven currents transporting drifting reproductive thalli fragments. As a selfing hermaphrodite species, surface wind-driven transportation of reproductive thalli can potentially start new populations at scattered sites even from a dispersal event of a single individual. A non-exclusive alternative explanation for the south-north boundary is counter-selection by physical or biological factors eliminating those that disperse beyond their current range.

Our data suggest that *F. spiralis* has been isolated in its South range from its other populations and in allopatry with *F. vesiculosus* for a period long enough for the observed levels of differentiation to take place. We thus propose that vicariance is promoting genetic distance between *F. spiralis* South and *F. spiralis* Low and High. Nevertheless, one question remains to be answered: Can this Southern entity reproduce successfully with any of the other *F. spiralis* entities and/or with *F. vesiculosus* or are there pre-zygotic reproductive barriers, such as gametic incompatibility, as in gamete recognition interactions? Answering this question would require experimental manipulations, as the absence of intermediate genotypes between allopatric entities could be simply due to geographic barriers precluding gene flow (pre-mating reproductive barriers).

Surprisingly, in two populations located at the southermost limit of the northern clade distribution area (North Spain and Brittany) the only type found was *F. spiralis* South. The dominant mtDNA haplotype was the same for both populations and it is one mutational step away from the most widely distributed South haplotype (h5) that was also found in one individual in Perharidy. This suggests some more recent connection between these Southern and the Northern locations. Furthermore, in one individual in Castello, we found a North Clade haplotype, sustaining the hypothesis of a recent North-South admixture in these regions. We propose that at least some few northern locations have been at some point colonized by some *F. spiralis* South.

It is noteworthy that *F. spiralis* South occurs mainly in allopatry, where *F. vesiculosus* is absent, and even where present further North, at least in Castello (North Spain) there is no *F. vesiculosus* present. This raises the question of whether *F. spiralis* South was present in several more Northern populations, but became diluted by high hybridization and introgression processes, with local *F. vesiculosus*. An alternative hypothesis would be that *F. spiralis* South is not present where *F. vesiculosus* occurs not by restricted dispersal but rather due to competitive exclusion, given that they share a vertical zone of the intertidal rocky shore.

The North Clade: F. spiralis *Low and* F. spiralis *High*

The "North entities", *F. spiralis* Low and *F. spiralis* High, were previously described based on microsatellite data where high hybridization rates, assessed as proportion of individuals with intermediate genotypes (because no species diagnostic markers exist), were observed between *F. spiralis* Low and *F. vesiculosus* but not between *F. spiralis* Low and *F. spiralis* High (Billard, 2007). This tendency was justified by the spatial proximity and a temporal discrepancy in females maturation between the high and the low intertidal zones that would favor inter-specific crosses while limiting vertical gene flow between individuals of the same species (Billard, 2007).

Conversely, when we analyze one of the populations described by Billard (2007), Santec, with our 12-microsatellite loci we did not detect such high hybridization rate between the two species, as very few intermediate genotypes could be found. However, the mitochondrial data shows a closer relationship between all haplotypes of *F. spiralis* Low and *F. vesiculosus* than between *F. spiralis* Low and *F. spiralis* High. For example, there is a shared haplotype (h7) between the two species, whereas none was found between the different entities within the species. In *F. spiralis,* this haplotype h7 was found in two Brittany populations, Santec and Port Lazo that are just few kilometers apart. In *F. vesiculosus,* it is widely present from South Portugal to the Faroes Islands and considering all haplotypes found in both species, h7 was pointed out by TCS software as the ancestral one. Despite this extensively shared haplotype, individuals of each species were well differentiated by microsatellite multilocus genotypes.

Several closely related and monophyletic *Fucus* species are accepted to have had a recent radiation (Serrão et al., 1999; Coyer et al., 2006) and it has been proposed that they may not be completely isolated both based on intermediate phenotypes (Hamel, 1939), unsorted lineages (Serrão et al., 1999; Coyer et al., 2006) and intermediate genotypes (Wallace et al., 2004; Engel et al., 2005; Billard, 2007). The direct interpretation of intermediate genotypes as single evidence for hybridization between these species has however been questioned (Engel et al., 2006). Nevertheless, one of the mechanisms proposed for speciation is through hybridization (Turelli et al., 2001; Ridley, 1996).

Lower hybrid fitness is one common explanation for the maintenance of species differentiation despite incomplete reproductive isolation. However, some of the introgression and combinations inherited could be favorable or even the best features from each parental species (Barton, 2001). But how can hybrids maintain their novel genotypes, untested by natural selection? One of the ways is by reproductive isolation from the parental species, which can be achieved, for instance by selfing, and/or by colonizing a new ecological niche (Turelli et al., 2001).

Both these characteristics are observed in some North populations of the hermaphroditic *F. spiralis*, which can reproduce by selfing and where some individuals, (*F. spiralis* Low) occupied a distinct niche, the mid-low intertidal zone. Therefore we can draw a possible scenario for the emergence of this *F. spiralis* Low entity presenting a mitochondrial haplotype shared with *F. vesiculosus*: a mitochondrial haplotype was donated by *F. vesiculosus* to an *F. spiralis* hybrid that afterwards reproduces by selfing. We can even hypothesize that this new combination was better adapted to the low intertidal than the original one from *F. spiralis*, the descendants inherited that haplotype that subsequently passed on to further generations.

Additionally, it is of interest to observe that the mtDNA haplotypes found in *F. spiralis* Low individuals are always private and unique haplotypes in each population. Here, the above argument is again valuable. Furthermore, looking at Hogerdijk (2008) data we can see that *F. vesiculosus* populations of the same or nearby sites, where *F. spiralis* Low were identified have the h7 haplotype in more than 50% of the individuals. So, we can argue that this mtDNA haplotype was inherited from *F. vesiculosus*, it since suffered some evolutionary mutations and has been maintained mainly by selfing.

But our nuclear data does not show this close inter-specific relationship between *F. spiralis* Low and *F. vesiculosus,* nor between *F. spiralis* Low and *F. spiralis* High. Hybridization was actually almost absent suggesting that they are apparently becoming isolated. Despite this hypothesis we must keep in mind that our microsatellite loci are EST-derived and at least some of them may be linked to genes under selection. In theory if selection is strong, then the natural tendency is to recover the parental genetic combination (Turelli et al., 2001). This way, while new well-adapted gene combinations are formed by selective pressure, at the same time high gene flow at neutral loci is still present (Buerkle et al., 2000). This might explain the observation of such extensive hybridization in Billard's (2007) work and so little in ours.

At this point we can differentiate these two northern *F. spiralis* entities genetically, they correspond in general to different morphological forms (Scott et al., 2001; Garreta et al., 2001) and physiological experiments to infer whether they are physiologically distinct are in progress (Zardi, Nicastro et al. in progress).

Conflicting Observations

Some cases of contradictory findings between nuclear and mtDNA data were observed in Port Lazo, one case in New Brighton and another in Albufeira. Although multilocus genotypes cluster these individuals with a certain entity, the mtDNA haplotype found was the most common one found in another entity. But it is expected that ancient introgression events continue to be detected as recombined genotypes in the recent history of the nuclear genome, whereas the mitochondria, by not recombining, exhibit better resolution for such distant sorting events (Zink & Barrowclough, 2008) that are transmitted intact albeit not yet complete in the nuclei. Under our hypotheses of an undergoing speciation process, the occasional occurrence of such conflicting genotype matches is a likely scenario.

The High/Low entities were not found in every North population, only between North Iberia and Brittany (plus Morocco, see below). On the one hand, and as pointed before, most of the populations in the North were collected using a horizontal transect along the typical area of most abundance of *F. spiralis* (sampling method a), therefore there might be some artifact in this absence of *F. spiralis* Low in these populations as such samples were not targeting the very low shore *F. spiralis*. Yet, in a previous study, we can see that even when North France was sampled in parapatric versus sympatric sites, only one entity was found, whereas with those same microsatellites the parapatric versus sympatric sites in Viana do Castelo, North Portugal, were clearly highly distinct (Engel et al., 2005). On the other hand, there are other healthy *F. spiralis* populations, like the case of New Brighton and Santoña, sharing the intertidal with *F. vesiculosus* that do not present a *F. spiralis* distinction even when sampled as Low (meaning as close to *F. vesiculosus* as possible) and High. Neither nuclear or mitochondria data differentiate these individuals sampled as Low and High. One possible explanation is the age of each population (Ridley, 1996). Viana do Castelo and Brittany may represent older populations where *F. spiralis/F. vesiculosus* hybrids (*F. spiralis* Low) had time to start to differentiate from the original *F. spiralis* as they are in an ecologically distinct environment to which they might now be adapted with inherited *F. vesiculosus* features.

In Morocco, a perfect match between both markers used was observed. Four individuals were identified as *F. spiralis* Low based on their genotype and a private haplotype was recognized. This is a curious observation, not only due to the non-sympatric occurrence of *F. spiralis* and *F. vesiculosus,* but also because it was the only Southern population displaying a North Clade haplotype. In its southern distribution range, *F. vesiculosus* is only found in estuarine habitats while *F. spiralis* is coastal (Ladah et al., 2003). In a *F. vesiculosus* phylogeography study (Hogerdijk, 2008) just one haplotype was found in the southernmost population, Ayamonte, and it is the one shared with its sister *F. spiralis*. Despite their estuarine distribution an older connectivity event between the open coast *F. spiralis* and estuarine *F. vesiculosus* is the most likely scenario for this pattern observed in Morocco. The recording of a *F. spiralis* northern haplotype just one mutation step away from the one shared with *F. vesiculosus* might indicate an ancient hybridization between the two species in this region even though they are now spatially segregated.

Besides the h7 haplotype, two other North Clade haplotypes were found in *F. spiralis* and *F. vesiculosus*: h1 and h12. The first, h1, was the most widely distributed in *F. spiralis* and only present in *F. vesiculosus* in two individuals in Cap Griz Nez and one individual in the Faroes Islands. The second, h12, was the most widely distributed haplotype in *F. vesiculosus*, dominating northern populations from Cornwall, Norway, the Baltic Sea, Iceland and the Western Atlantic. Regarding this discrepancy in their distribution and the known difficulties of identification in the field of these challenging species we consider it likely that there was a misidentification during sampling campaigns.

Phylogeography

In the last years, the evolutionary history of North Atlantic marine taxa has been a topic of interest in research (Maggs et al., 2008 for review). One of our goals was to recognize possible glacial refugia and post-glacial recolonization pathways of the amphi-Atlantic seaweed *F. spiralis*. However, the attempt to reconstruct the phylogeography of *F. spiralis* now appears somewhat complex given that, as discussed above, we are apparently uncovering an intricate and unsolved history between *F. spiralis* and *F. vesiculosus*. Clearer results were obtained using similar methods for *F. serratus* (Hoarau et al., 2007b) and *F. vesiculosus* (Hogerdeijk, 2008) revealing similar phylogeographic histories, including the proposal of suitable glacial refugia and consequent recolonization pathways. Glacial refugia for these species have been proposed in the region of Brittany, where populations show the highest diversity levels currently found, but perhaps also North Iberia due to the presence of unique haplotypes.

Although glacial refugia are often identified as having high diversity values and private alleles/haplotypes (Hewitt, 2001), such high diversity might not be encompassed by single populations but rather by considering the whole set of populations of the species in a given region, because populations in such refugial areas, often currently small and isolated, might be very distinct from each other (Petit et al., 2003). Such differentiation might arise due to isolation between small populations resulting in low gene flow and accelerated genetic drift.

Two Glacial Refugia

The large differences observed between South and North *F. spiralis* populations might be an example of the processes mentioned above. As discussed above the current maintenance of the genetic break between South and North of the Iberian West Coast might be due to barriers to gene flow and/or selective processes. This South/North break has been observed in other species (Alberto et al., 1999; Gomes et al., 2001; Diekmann et al., 2005) and recent and ongoing work shows the same pattern for these and other *Fucus* species (Daguin in prep, Perrin in prep, Neiva, in progress). However, the main event that originated this separation into two groups might be associated with two distinct glacial refugia that have left imprint marks on several taxa. Therefore, we suggest that South Iberia, Morocco, Canary Islands and Azores, which were ice-free areas during the LGM, functioned as a refugial area for *F.spiralis* distinct and separate from the one for the *F. spiralis* populations that recolonized the North.

Southern Populations

In the Southern range, the mtDNA high diversity levels found in the Azores suggest the hypothesis that isolation of multiple populations in different Azorean islands might have allowed differentiation of distinct populations that diverged also in relation to the mainland populations, as might be expected for distant islands as areas with high rates of genetic drift and extremely restricted, if any, interpopulation gene flow (Ridley, 1996). If we would follow the theoretical recognition of glacial refugia as regions of high diversity because they were able to maintain the species occurring for extended periods, we could actually propose the Azores as one such area for *F. spiralis* South. Nevertheless, the whole South was completely ice-free, and although it was much colder, *F. spiralis* is a cold-adapted species, well capable of surviving even through freezing conditions (Davidson & Pearson, 1996), so, it is not expected that one specific geographical area in the south served as refugium, given that these organisms could potentially survive in the entire area, as long as the shoreline had rocky substrate.

Northern Populations

The extremely low diversities in the Northern range suggest that they were recolonized through a founder event from a southern population, as the ice retreated after the LGM. We suggest that Iberia and/or Brittany are the most likely areas to have acted as source for these recolonization events. Besides being nominated as glacial refugia for several other marine and terrestrial species (Hewitt, 2000; Maggs et al., 2008), in these areas we found that all three entities occur, and it includes the highest diversity levels, thus encompasses the main diversity that exists within the species.

The unexpected occurrence of *F. spiralis* South in these regions (North Iberia and Brittany) may be due to colonization from the South taking place after the divergence of the North/South entities. As noted by Petit (2003), divergent populations might be maintained by isolation from neighbouring populations, especially if we are dealing with a preferentially selfing species that needs only one individual to successfully colonize an area while maintaining its genetic composition in the new population. However, it is still odd that among all northern populations only these two displayed South Clade haplotypes, particularly because they were found in every individual, with the exception of one in Castello. We hypothesize that these South Clade individuals may be better adapted to southern conditions of higher physical stress, such as higher desiccation periods, photoinhibitory irradiances, or higher temperatures and that in northern distributional ranges they may be less competitive. Accordingly, we may then also hypothesize that the Brittany region is at their north latitudinal limit, thereby explaining why Southern clade haplotypes were not found in any locations further North.

The USA Atlantic coast also displays low diversity, and the exact same nuclear and mitochondrial haplotypes as in northern Europe, which corroborates the hypothesis of the West Atlantic intertidal having been devastated during the LGM, with recolonization having occurred from the Eastern Atlantic populations as it ended (Wares & Cunningham, 2001).

Serrão et al. (1999) and Coyer et al. (2006) proposed that the discrete populations of *F. spiralis* in the USA Pacific coast were probably a human introduction. Natural colonization events or as origin of the lineage are very unlikely scenarios as there are few scattered populations (Coyer et al., 2006). The presence of a unique haplotype in the Pacific population, shared with Atlantic populations, supports the hypothesis of a recent colonization of the Pacific by *F. spiralis*.

Conclusions

Major Conclusions

- *F. spiralis* includes three most common divergent entities, one mainly in its southern range (named South), another one mainly in its northern range (named High), and a third northern entity has also been found in some populations mostly between North Iberia and Brittany (southernmost area of the northern type range), where it occurs lower on the shore. The latter has also been found in Morocco. We hypothesize this was derived from ancient introgression with *F. vesiculosus*.
- The northern range types (High and Low) share, or are similar to, mitochondrial haplotypes of *F. vesiculosus*, particularly true of the Low type.
- The High and Low entities are parapatric, with a broad contact zone, yet the lack of intermediate genotypes suggest reproductive isolation, and consequent ongoing speciation processes.
- The separation between a southern and a northern type suggests a long period of isolation in two distinct geographical regions, we thus hypothesize these as two distinct glacial refugia, one in the southern range and another one in the Brittany-North Iberia range.
- Most of the north Atlantic, the West Atlantic and the Pacific appear to have been recolonized from a unique source from the High shore type.

Concluding Remarks

A take home lesson of this study is to never take for granted the biological patterns assumed from previous studies and always expect that novel data may prove our previous hypothesis to be false. The genus *Fucus* remains a challenging group in terms of phylogeny, ecology and speciation. Although it has been widely used as a model marine taxon in ecological, physiological and developmental research for over a century, it continues to surprise us. One thing is clear though: *F. spiralis* is much more complex than a simple, homogeneous, high intertidal seaweed species that can hybridize with *F. vesiculosus*. In order to clarify the *F. spiralis* story, further work should be conducted in ecology, physiology and reproductive biology in order to understand the degree of reproductive isolation and selective differences between these three entities. In addition, other molecular markers should be used in large-scale geography sampling to develop an unambiguous phylogeny of *F. spiralis* and the genus *Fucus*.

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APPENDICES

APPENDIX A

New Streamlined CTAB method of Extraction from *Fucus/Zostera*

1. Use 5-10 mg of silica-dried sample
2. Place in 1.2 mL plastic tubes with steel ball (large)
3. Grind:
   a. Time = 10 seconds
   b. Frequecy = 25
4. Remove steel balls with magnet
5. Add 400 uL CTAB and β mecapethanol
6. Gently rotate for 1hr or so at room temperature for *Fucus*; 55-60º C for *Zostera*
7. Add 400 uL CIA (24:1, chloroform: isoamyl alcohol)
8. Centrifuge 3000 rpm for 20 min
9. Assemble filter plate (Milipore MultiScreen HTS, FB Cat. #MSFBN6B10): silica filter
   a. Add 150 uL NaI (gel solubizing buffer)
   b. Add 150 uL of aqueous extract from ‘8’
10. Centrifuge using waste plate
    a. 1000 rpm for 15 min
    b. 2000 rpm for 10 min
11. Wash 1x with 150 uL wash buffer (do not mix): use waste plate
12. Centrifuge 3000 rpm for 10 min (use waste plate)
13. Set plate to dry for 30 min
14. Elute:
    a. 100 uL 0.1X TE at 55º C
    b. Incubate for 5 min
15. Centrifuge using sample titer plate
    a. 1000 rpm for 5 min
    b. 2000 rpm for 5 min

Solutions:

	For 1 Liter
1. CTAB extraction buffer	
a. 100 mM Tris-HCl pH 8.0	12.11 g
b. 1.4 M NaCl	81.82 g
c. 20 mM EDTA	7.44 g
d. Adjust pH to pH = 8.	
e. Heat in microwave to ca 60º C before adding CTAB in order to get CTAB into solution	
f. 2% (w/v) CTAB	20.00 g
g. 0.1% (w/v) PVPP	1.00 g
h. *0.2% (v/v) β mecapethanol (add fresh daily)*	

2. Wash Buffer (to be prepared for each plate)	
a. Stock Wash Buffer	4.2 mL
b. 100% EtOH	10.8 mL
c. Stock Wash Buffer (100mL)	
a. 2mL 1M Tris (pH 8.0)	
b. 0.2 mL 0.5M EDTA	
c. 2.5 uL 4M NaCl	
d. Deionized water to 100mL	

APPENDIX B

Cleaning products with Sephadex®

1. Use the SigmaSpin 2 Post-reaction clean-up plates
2. Add 5g Sephadex G-50 to 75 mL of milliQ water and incubate for 10 min
3. Add 50 µL of milliQ water into the wells, than add 750 µL of the Sephadex slurry
4. Put the plate on a waste plate and centrifuge for 2 min at 3000 rpm
5. Empty the waste plate and put it back
6. Centrifuge again for 2 min at 3000 rpm
7. Remove the waste plate and put the plate with Sephadex on a clean microtiterplate
8. Load the PCR-product or sequence reaction
9. Centrifuge for 3 min at 3000 rpm
10. Dry the cleaned samples in a speedvac at medium temperature (45°C) *(only for sequence reactions)*

APPENDIX C

Cleaning PCR products with ExoSapIt enzyme (for sequencing)

1. Check on an agarose gel th amount of PCR product needed for sequencing
2. Put the amount needed (10 – 20 ng/uL) for each sequencing reaction in a 96 well plate
3. Dry the samples in the speedvac at medium heat (45°C)
4. Add 5,2 µL of milliQ and 1,8 µL of enzyme in each well
5. Run program as follows:
    a. 37° for 15 min, than 80°C for 15 min, (4°C as dwell temperature)

APPENDIX D

Settings on ABI 3130XL for SSCP

Oven temperature	24°C
Poly_Fill_Vol	6500 steps
Current Stability	5,0 uAmps
PreRun Voltage	10,0 kV
PreRun Time	220 s
Injection Voltage	5,0 kV
Injection Time	25 s
Voltage Number Of Steps	40 nk
Voltage Step Interval	15 s
Data Delay Time	1 s
Run Voltage	10,0 kV
Run Time	240

APPENDIX E

```
                  10        20        30        40        50        60        70        80        90       100
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
fs h1     TCCTATTGGTTATTTATAACGATAGTCGTTTAAATGCTACACTCCTAACCCAGTTAAAATTGATGGGTTAGTTATTTAATGGTCTTTAGAATAAAATTCA
fs h2     ........................T...................T.......................................................
fs h3     ....................................................................................................
fs h4     ..........................T...................................G.....................................
fs h5     ..............................................................G.....................................
fs h6     ..............................................................G.....................................
fs h7     ....................................................................................................
fs h8     ..............................................................G.....................................
fs h9     ..............................................................G...................................T.
fs h10    ....................................................................................................
fs h11    ..................................................................................C.................
fs h12    .................................................................................................C..
fs h13    ....................................................................................................
fs h14    ....................................................................................................
fs h15    ....................................................................................................
fs_h16    ....................................................................................................

                 110       120       130       140       150       160       170       180       190       200
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
fs h1     ATTTCAACTAAAAATATTCCTCTCATCAAACGTCTACTTTTTTACGTCCAAAAAGACGCGTTGTTTTAGAGGGTAGCGCAGTTTATTAACGTTATATCTT
fs h2     ...............................A.................................C..................................
fs h3     ...............................A.................................C..................................
fs h4     ..............................T...........................T......C.............G....................
fs h5     ..............................T...........................T......C.............G....................
fs h6     ..............................T...........................T....................G....................
fs h7     .................................................................C..................................
fs h8     ................C.............T...........................T......C..................................
fs h9     ................C.............T...........................T......C..................................
fs h10    .........................C..........................................................................
fs h11    ....................................................................................................
fs h12    .................................................................C..................................
fs h13    .............................................................................A......................
fs h14    ..A.................................................................................................
fs h15    ...............................................................................................G....
fs_h16    .................................................................C.............G....................

                 210
          ....|....|
fs h1     TTAGAAAATG
fs h2     ..........
fs h3     ..........
fs h4     ..........
fs h5     ..........
fs h6     ..........
fs h7     ..........
fs h8     ..........
fs h9     ..........
fs h10    ..........
fs h11    ..........
fs h12    ..........
fs h13    ..........
fs h14    ..........
fs h15    ..........
fs_h16    ..........
```

Fig. E1. Nucleotide Sequences of the 210 bp mt-IGS found in 733 *F. spiralis* individuals around its entire distribution range.

APPENDIX F

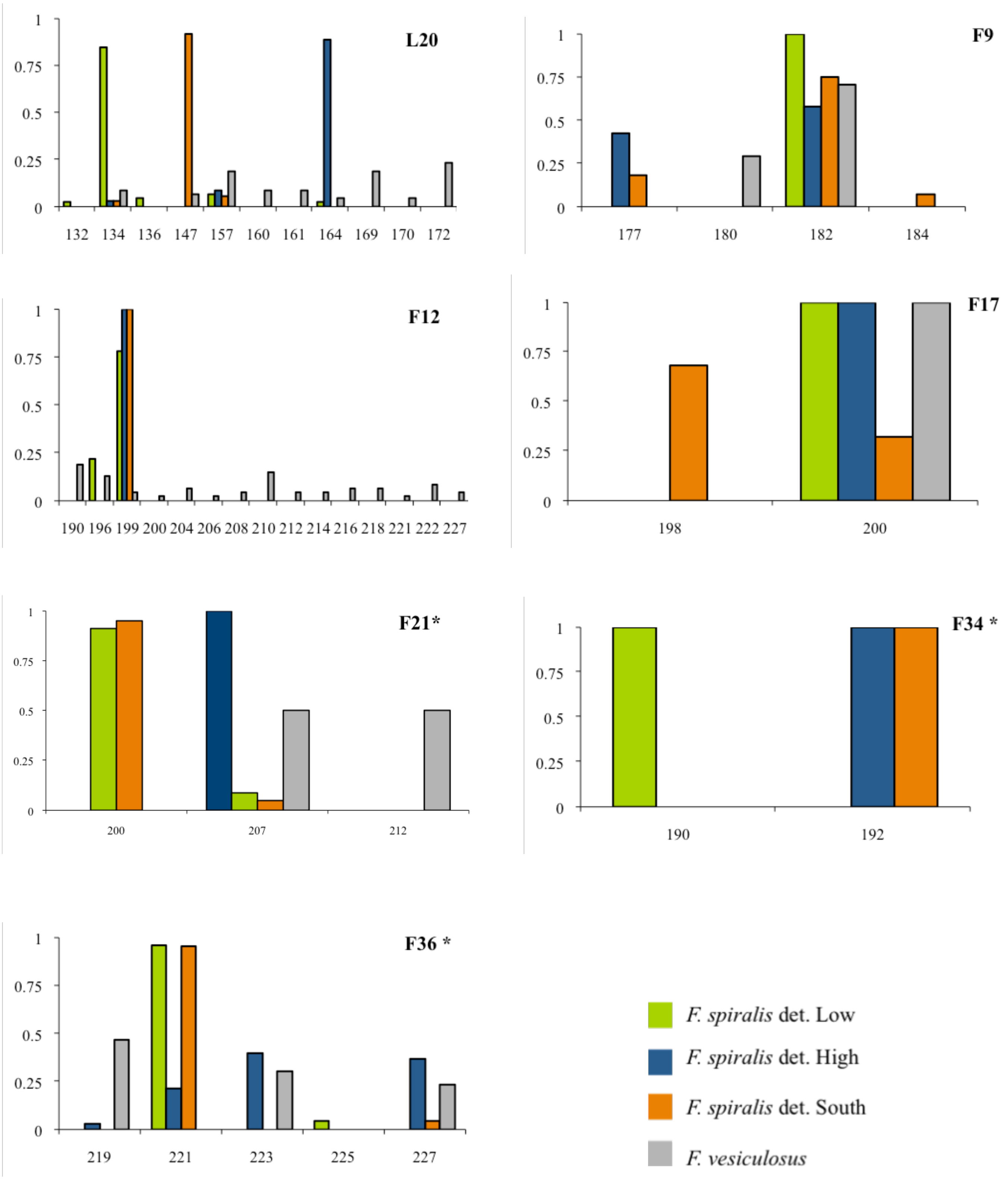


Fig. F1. Allelic frequencies of the 13 microsatellite loci amplified to distinguish the different *F. spiralis* entities and *F. vesiculosus* for Santec and Perharidy populations. * locus of the 5 subset used as diagnostical for all the others locations genotyped. F34 was excluded for the analysis with *F. vesiculosus*

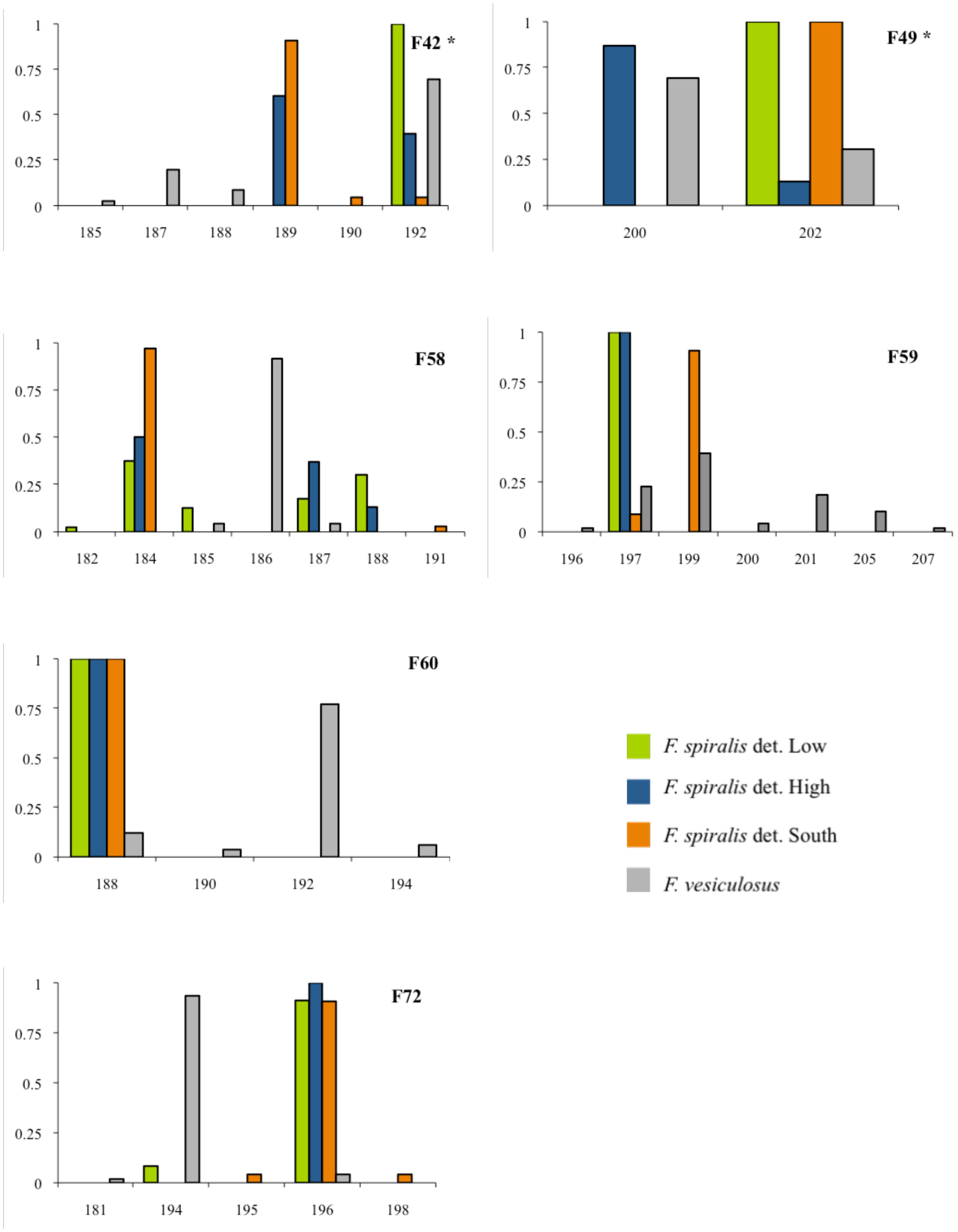
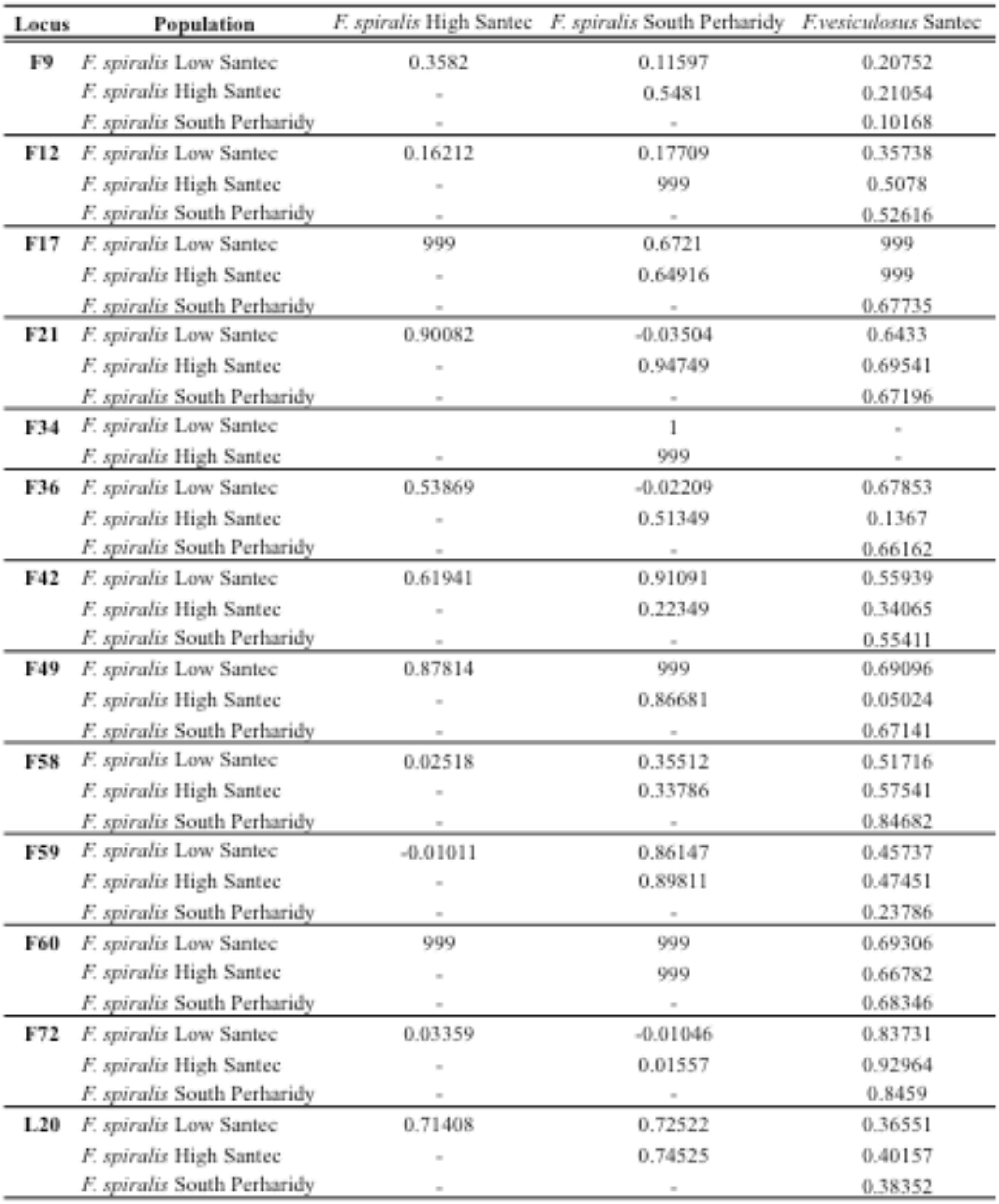


Fig. F1 (cont.). Allelic frequencies of the 13 microsatellite loci amplified to distinguish the different *F. spiralis* entities and *F. vesiculosus* for Santec and Perharidy populations. * locus of the 5 subset used as diagnostical for all the others locations genotyped. F34 was excluded for the analysis with *F. vesiculosus*

Table FI. Pairwise population Fst values (Weir & Cockerham, 1984) for the 13 microsatellites loci calculated with Genetix software.



Locus	Population	*F. spiralis* High Santec	*F. spiralis* South Perharidy	*F.vesiculosus* Santec
F9	*F. spiralis* Low Santec	0.3582	0.11597	0.20752
	F. spiralis High Santec	-	0.5481	0.21054
	F. spiralis South Perharidy	-	-	0.10168
F12	*F. spiralis* Low Santec	0.16212	0.17709	0.35738
	F. spiralis High Santec	-	999	0.5078
	F. spiralis South Perharidy	-	-	0.52616
F17	*F. spiralis* Low Santec	999	0.6721	999
	F. spiralis High Santec	-	0.64916	999
	F. spiralis South Perharidy	-	-	0.67735
F21	*F. spiralis* Low Santec	0.90082	-0.03504	0.6433
	F. spiralis High Santec	-	0.94749	0.69541
	F. spiralis South Perharidy	-	-	0.67196
F34	*F. spiralis* Low Santec		1	-
	F. spiralis High Santec	-	999	-
F36	*F. spiralis* Low Santec	0.53869	-0.02209	0.67853
	F. spiralis High Santec	-	0.51349	0.1367
	F. spiralis South Perharidy	-	-	0.66162
F42	*F. spiralis* Low Santec	0.61941	0.91091	0.55939
	F. spiralis High Santec	-	0.22349	0.34065
	F. spiralis South Perharidy	-	-	0.55411
F49	*F. spiralis* Low Santec	0.87814	999	0.69096
	F. spiralis High Santec	-	0.86681	0.05024
	F. spiralis South Perharidy	-	-	0.67141
F58	*F. spiralis* Low Santec	0.02518	0.35512	0.51716
	F. spiralis High Santec	-	0.33786	0.57541
	F. spiralis South Perharidy	-	-	0.84682
F59	*F. spiralis* Low Santec	-0.01011	0.86147	0.45737
	F. spiralis High Santec	-	0.89811	0.47451
	F. spiralis South Perharidy	-	-	0.23786
F60	*F. spiralis* Low Santec	999	999	0.69306
	F. spiralis High Santec	-	999	0.66782
	F. spiralis South Perharidy	-	-	0.68346
F72	*F. spiralis* Low Santec	0.03359	-0.01046	0.83731
	F. spiralis High Santec	-	0.01557	0.92964
	F. spiralis South Perharidy	-	-	0.8459
L20	*F. spiralis* Low Santec	0.71408	0.72522	0.36551
	F. spiralis High Santec	-	0.74525	0.40157
	F. spiralis South Perharidy	-	-	0.38352

APPENDIX G

Table GI. Alleles found in the 5 EST-derived msat (Coyer et al., 2008) in seven populations of *F. spiralis*.

Location	Individual	Collected as	Locus				
			F21	F34	F36	F42	F49
New Brighton, Liverpool, England	1548	Low	207207	192192	221221	189189	202202
New Brighton, Liverpool, England	1549	Low	207207	192192	221221	189189	202202
New Brighton, Liverpool, England	1550	Low	207207	192192	223227	189189	202202
New Brighton, Liverpool, England	1551	Low	207207	192192	221221	189189	202202
New Brighton, Liverpool, England	1552	Low	207207	192192	221221	189189	200200
New Brighton, Liverpool, England	1553	Low	207207	192192	227227	189189	202202
New Brighton, Liverpool, England	1554	Low	207207	192192	221221	189189	202202
New Brighton, Liverpool, England	1555	Low	207207	192192	221221	192192	200200
New Brighton, Liverpool, England	1556	Low	207207	192192	227227	189189	202202
New Brighton, Liverpool, England	1557	Low	207207	192192	221221	192192	202202
New Brighton, Liverpool, England	1558	Low	207207	192192	227227	192192	200200
New Brighton, Liverpool, England	1559	Low	207207	192192	227227	192192	200200
New Brighton, Liverpool, England	1560	Low	207207	192192	221221	192192	200200
New Brighton, Liverpool, England	1561	Low	207207	192192	221221	189189	202202
New Brighton, Liverpool, England	1562	Low	207207	192192	227227	189189	202202
New Brighton, Liverpool, England	1563	Low	207207	192192	221221	192192	202202
New Brighton, Liverpool, England	1564	Low	207207	192192	227227	189189	200200
New Brighton, Liverpool, England	1565	Low	207207	192192	227227	189192	202202
New Brighton, Liverpool, England	1566	Low	207207	192192	227227	189189	202202
New Brighton, Liverpool, England	1567	Low	207207	192192	227227	189189	200200
New Brighton, Liverpool, England	1568	Low	207207	192192	227227	189189	202202
New Brighton, Liverpool, England	1569	Low	207207	192192	223223	189189	202202
New Brighton, Liverpool, England	1570	Low	207207	192192	221221	189189	202202
New Brighton, Liverpool, England	1571	Low	207207	192192	221221	189189	202202
New Brighton, Liverpool, England	1598	High	207207	192192	221221	192192	202202
New Brighton, Liverpool, England	1599	High	207207	192192	227227	192192	200200
New Brighton, Liverpool, England	1600	High	207207	192192	221221	192192	202202
New Brighton, Liverpool, England	1601	High	207207	192192	221221	189189	202202
New Brighton, Liverpool, England	1602	High	207207	192192	227227	192192	202202
New Brighton, Liverpool, England	1603	High	207207	192192	227227	189189	202202
New Brighton, Liverpool, England	1604	High	207207	192192	221221	189189	200200
New Brighton, Liverpool, England	1605	High	207207	192192	221221	189189	202202
New Brighton, Liverpool, England	1606	High	207207	192192	221221	189189	202202
New Brighton, Liverpool, England	1607	High	200200	0	0	192192	0
New Brighton, Liverpool, England	1608	High	207207	192192	221221	192192	202202
New Brighton, Liverpool, England	1609	High	207207	192192	221221	192192	202202
New Brighton, Liverpool, England	1610	High	207207	192192	221221	189189	202202
New Brighton, Liverpool, England	1611	High	207207	192192	221221	189189	202202
New Brighton, Liverpool, England	1612	High	207207	192192	221221	189189	200200
New Brighton, Liverpool, England	1613	High	207207	192192	221221	192192	202202
New Brighton, Liverpool, England	1614	High	207207	192192	221221	189192	202202
New Brighton, Liverpool, England	1615	High	207207	192192	221221	192192	200200
New Brighton, Liverpool, England	1616	High	207207	192192	221221	189189	200200
New Brighton, Liverpool, England	1617	High	207207	192192	227227	189189	202202
New Brighton, Liverpool, England	1618	High	207207	192192	221221	192192	202202
New Brighton, Liverpool, England	1619	High	207207	192192	227227	192192	202202
New Brighton, Liverpool, England	1620	High	207207	192192	227227	192192	202202
New Brighton, Liverpool, England	1621	High	207207	192192	221221	189189	202202
Port Lazo, Brittany, France	1384	Low	200200	190190	221221	192192	202202
Port Lazo, Brittany, France	1385	Low	200200	190190	221221	189189	202202

Location	Individual	Collected as	Locus				
			F21	F34	F36	F42	F49
Port Lazo, Brittany, France	1386	Low	200200	190194	221221	189189	202202
Port Lazo, Brittany, France	1387	Low	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1388	Low	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1389	Low	207207	190194	221227	189192	200202
Port Lazo, Brittany, France	1390	Low	200200	190190	221221	189192	202202
Port Lazo, Brittany, France	1391	Low	200200	190190	223223	192192	202202
Port Lazo, Brittany, France	1392	Low	200200	190190	221221	189192	202202
Port Lazo, Brittany, France	1393	Low	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1394	Low	200200	190194	221221	189189	202202
Port Lazo, Brittany, France	1395	Low	200200	190194	221221	189192	202202
Port Lazo, Brittany, France	1396	Low	200200	189189	221221	189189	202202
Port Lazo, Brittany, France	1397	Low	200200	190190	221221	192192	202202
Port Lazo, Brittany, France	1398	Low	200200	190190	223223	189192	202202
Port Lazo, Brittany, France	1399	Low	200200	190190	221221	189192	202202
Port Lazo, Brittany, France	1400	Low	200200	190190	221221	189192	202202
Port Lazo, Brittany, France	1401	Low	200200	190190	221221	189192	202202
Port Lazo, Brittany, France	1402	Low	207207	190194	221221	189189	200200
Port Lazo, Brittany, France	1403	Low	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1404	Low	200200	190190	223223	189189	202202
Port Lazo, Brittany, France	1405	Low	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1406	Low	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1407	Low	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1415	High	207207	192192	223223	189189	202202
Port Lazo, Brittany, France	1416	High	207207	192192	223223	189189	202202
Port Lazo, Brittany, France	1417	High	207207	192192	221221	189189	200200
Port Lazo, Brittany, France	1418	High	207207	190192	221221	189189	200200
Port Lazo, Brittany, France	1419	High	200200	192192	223223	189192	202202
Port Lazo, Brittany, France	1420	High	207207	190190	221221	189189	202202
Port Lazo, Brittany, France	1421	High	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1422	High	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1423	High	200200	190192	221221	189189	202202
Port Lazo, Brittany, France	1424	High	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1425	High	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1426	High	207207	192192	221223	189189	200200
Port Lazo, Brittany, France	1427	High	207207	192192	223223	189189	202202
Port Lazo, Brittany, France	1428	High	0	190190	221221	189189	202202
Port Lazo, Brittany, France	1429	High	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1430	High	200200	190190	221221	189189	202202
Port Lazo, Brittany, France	1431	High	200200	192192	221221	189189	202202
Port Lazo, Brittany, France	1432	High	207207	190190	223223	189189	202202
Port Lazo, Brittany, France	1433	High	207207	192192	223223	189192	202202
Port Lazo, Brittany, France	1434	High	207207	190190	221221	189192	0
Port Lazo, Brittany, France	1435	High	200200	190190	221221	189192	202202
Port Lazo, Brittany, France	1436	High	207207	192192	223223	192192	202202
Port Lazo, Brittany, France	1437	High	207207	192192	223227	189189	202202
Port Lazo, Brittany, France	1438	High	200200	190190	221221	192192	202202
Santec, Brittany, France	0a	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	0b	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	10a	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	13b	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	14a	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	15b	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	16a	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	16b	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	17a	Low	200200	190190	221221	192192	202202

Location	Individual	Collected as	Locus				
			F21	F34	F36	F42	F49
Santec, Brittany, France	19b	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	1a	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	20a	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	20b	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	2b	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	3a	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	4a	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	5b	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	6a	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	6b	Low	0	190190	221221	192192	202202
Santec, Brittany, France	7b	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	8a	Low	207207	190190	221221	192192	202202
Santec, Brittany, France	8b	Low	200200	190190	221221	192192	202202
Santec, Brittany, France	9b	Low	200200	190190	225225	192192	202202
Santec, Brittany, France	9b	Low	207207	190190	221221	192192	202202
Santec, Brittany, France	-10a	High	207207	192192	227227	189189	200202
Santec, Brittany, France	-11a	High	207207	192192	223223	189189	202202
Santec, Brittany, France	-12b	High	207207	192192	227227	189192	200200
Santec, Brittany, France	-13b	High	207207	192192	223223	192192	200200
Santec, Brittany, France	-13b	High	207207	192192	227227	192192	200200
Santec, Brittany, France	-14a	High	207207	192192	223223	189189	200200
Santec, Brittany, France	-14b	High	207207	192192	227227	189189	200200
Santec, Brittany, France	-15a	High	207207	192192	223227	192192	200200
Santec, Brittany, France	-15b	High	207207	192192	227227	189189	200200
Santec, Brittany, France	-16a	High	207207	192192	223223	192192	200200
Santec, Brittany, France	-16b	High	207207	192192	223223	189189	200200
Santec, Brittany, France	-17b	High	207207	192192	221221	189189	200200
Santec, Brittany, France	-17b	High	207207	192192	221227	189192	200202
Santec, Brittany, France	-18a	High	207207	192192	223223	192192	200200
Santec, Brittany, France	-18b	High	207207	192192	223223	189189	200200
Santec, Brittany, France	-19a	High	207207	192192	221221	189189	200200
Santec, Brittany, France	-19b	High	207207	192192	227227	189189	200200
Santec, Brittany, France	-20a	High	207207	192192	221221	189192	200202
Santec, Brittany, France	-20b	High	207207	192192	219221	192192	200200
Perharidy, Brittany, France	550	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	551	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	552	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	553	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	554	Unknown	200200	192192	227227	192192	0
Perharidy, Brittany, France	555	Unknown	200200	192192	227227	189189	0
Perharidy, Brittany, France	556	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	557	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	558	Unknown	207207	192192	221221	189189	202202
Perharidy, Brittany, France	559	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	560	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	561	Unknown	0	192192	221221	189189	202202
Perharidy, Brittany, France	562	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	563	Unknown	200200	192192	221221	189189	0
Perharidy, Brittany, France	564	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	565	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	566	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	567	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	568	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	569	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	570	Unknown	200200	192192	221221	189189	202202

Location	Individual	Collected as	Locus				
			F21	F34	F36	F42	F49
Perharidy, Brittany, France	571	Unknown	200200	192192	221221	189189	202202
Perharidy, Brittany, France	572	Unknown	0	192192	221221	189189	202202
Perharidy, Brittany, France	573	Unknown	200200	192192	221221	189189	202202
Santoña, Cantabria, Spain	1723	Low	207207	190190	223223	192192	202202
Santoña, Cantabria, Spain	1724	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1725	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1726	Low	207212	190192	227227	189192	200200
Santoña, Cantabria, Spain	1727	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1728	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1729	Low	207207	190192	223223	189192	200200
Santoña, Cantabria, Spain	1730	Low	207207	192192	223223	192192	200200
Santoña, Cantabria, Spain	1731	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1732	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1733	Low	207207	192192	223223	189189	200200
Santoña, Cantabria, Spain	1734	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1735	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1736	Low	207207	192192	223223	192192	200200
Santoña, Cantabria, Spain	1737	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1738	Low	207207	192192	223227	193193	200200
Santoña, Cantabria, Spain	1739	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1740	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1741	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1742	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1743	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1744	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1745	Low	207207	192192	223223	189189	200200
Santoña, Cantabria, Spain	1746	Low	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1750	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1751	High	207207	192192	223223	192192	202202
Santoña, Cantabria, Spain	1752	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1753	High	207207	192192	223223	192192	202202
Santoña, Cantabria, Spain	1754	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1755	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1756	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1757	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1758	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1759	High	207207	192192	223223	189189	200200
Santoña, Cantabria, Spain	1760	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1761	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1762	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1763	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1764	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1765	High	207207	192192	223223	192192	202202
Santoña, Cantabria, Spain	1766	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1767	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1768	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1769	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1770	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1771	High	207207	192192	223223	192192	202202
Santoña, Cantabria, Spain	1772	High	207207	192192	223223	189189	202202
Santoña, Cantabria, Spain	1773	High	207207	192192	223223	192192	202202
Viana do Castelo, Portugal	78a	Low	200207	192192	221221	192192	202202
Viana do Castelo, Portugal	60b	Low	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	59a	Low	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	59b	Low	207207	192192	221221	192192	202202

Location	Individual	Collected as	Locus				
			F21	F34	F36	F42	F49
Viana do Castelo, Portugal	47a	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	64b	Low	207207	192192	221221	189189	202202
Viana do Castelo, Portugal	68b	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	72b	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	76b	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	78b	Low	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	47b	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	62b	Low	200200	190190	223223	192192	200200
Viana do Castelo, Portugal	66b	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	70a	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	73b	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	77a	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	79a	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	49b	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	63a	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	67a	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	71b	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	74a	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	77b	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	79b	Low	200200	190190	223223	192192	202202
Viana do Castelo, Portugal	7	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	16	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	34	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	46	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	58	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	70	High	207207	192192	221221	189189	202202
Viana do Castelo, Portugal	82	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	Q5_100	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	10	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	25	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	37	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	49	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	61	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	73	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	85	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	1	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	13	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	28	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	52	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	64	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	76	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	4	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	22	High	207207	192192	221221	192192	202202
Viana do Castelo, Portugal	31	High	207207	192192	221221	192192	202202
El Medano, Tenerife, Canary Isl	1921	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1922	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1923	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1924	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1925	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1926	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1927	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1928	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1929	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1930	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1931	None	200200	192192	225225	189192	200200

Location	Individual	Collected as	Locus				
			F21	F34	F36	F42	F49
El Medano, Tenerife, Canary Isl	1932	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1933	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1934	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1935	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1936	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1937	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1938	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1939	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1940	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1941	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1942	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1943	None	200200	192192	225225	189189	200200
El Medano, Tenerife, Canary Isl	1944	None	200200	192192	225225	189189	200200
Albufeira, Portugal	700	None	200200	192192	227227	189189	202202
Albufeira, Portugal	701	None	200200	192192	227227	189189	202202
Albufeira, Portugal	702	None	200200	192192	227227	189189	202202
Albufeira, Portugal	703	None	200200	192192	227227	189189	202202
Albufeira, Portugal	704	None	207207	192192	0	189189	202202
Albufeira, Portugal	705	None	200200	192192	227227	189189	202202
Albufeira, Portugal	706	None	200200	192192	227227	189189	202202
Albufeira, Portugal	707	None	200200	192192	0	189189	0
Albufeira, Portugal	708	None	200200	192192	227227	189189	202202
Albufeira, Portugal	709	None	200200	192192	227227	189189	202202
Albufeira, Portugal	710	None	200200	192192	227227	189189	202202
Albufeira, Portugal	711	None	200200	192192	227227	189189	0
Albufeira, Portugal	712	None	200200	192192	227227	189189	202202
Albufeira, Portugal	713	None	200200	192192	227227	189189	202202
Albufeira, Portugal	714	None	200200	192192	227227	189189	202202
Albufeira, Portugal	715	None	207207	192192	225225	189189	0
Albufeira, Portugal	716	None	200200	192192	227227	189189	202202
Albufeira, Portugal	717	None	200200	192192	227227	189189	202202
Albufeira, Portugal	718	None	200200	192192	227227	189189	202202
Albufeira, Portugal	719	None	0	0	221221	189189	0
Albufeira, Portugal	720	None	200200	192192	227227	189189	202202
Albufeira, Portugal	721	None	200200	192192	227227	189189	202202
Albufeira, Portugal	722	None	200200	192192	227227	189189	202202
Albufeira, Portugal	723	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1264	None	200200	190190	223223	192192	202202
Oualidia, Morocco	1265	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1266	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1267	None	200200	192192	223223	189189	202202
Oualidia, Morocco	1268	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1269	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1270	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1271	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1272	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1273	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1274	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1275	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1276	None	200200	192192	227227	189189	202204
Oualidia, Morocco	1277	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1278	None	200200	190190	223223	189189	202202
Oualidia, Morocco	1279	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1280	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1281	None	200200	190190	223223	189189	202202

Location	Individual	Collected as	Locus				
			F21	F34	F36	F42	F49
Oualidia, Morocco	1282	None	200200	190190	223223	189189	202202
Oualidia, Morocco	1283	None	200200	192192	223223	189189	202202
Oualidia, Morocco	1284	None	200200	192192	223223	189189	202202
Oualidia, Morocco	1285	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1286	None	200200	192192	227227	189189	202202
Oualidia, Morocco	1287	None	200200	192192	227227	189189	202202

Table GII. Alleles present in 13 msat loci of *F. spiralis* and *F. vesiculosus* individuals.

Location	Individual	Collected as	Locus												
			L20	F9	F12	F21	F34	F36	F42	F49	F60	F17	F58	F59	F72
Santec, Brittany, France	0a	Low	134134	182182	199199	200200	190190	221221	192192	202202	188188	200200	185185	198198	196196
Santec, Brittany, France	0b	Low	134134	182182	199199	200200	190190	221221	192192	202202	188188	200200	184184	197197	196196
Santec, Brittany, France	10a	Low	134134	182182	199199	200200	190190	221221	192192	202202	188188	200200	0	197197	196196
Santec, Brittany, France	13b	Low	134134	182182	199199	200200	190190	221221	192192	202202	188188	200200	184188	197197	196196
Santec, Brittany, France	14a	Low	134134	182182	199199	200200	190190	221221	192192	202202	188188	200200	187187	197197	196196
Santec, Brittany, France	15b	Low	134134	182182	197197	200200	190190	221221	192192	202202	188188	200200	184188	197197	196196
Santec, Brittany, France	16a	Low	134134	182182	199199	200200	190190	221221	192192	202202	188188	200200	184184	197197	196196
Santec, Brittany, France	16b	Low	134134	182182	199199	200200	190190	221221	192192	202202	188188	200200	185188	197197	196196
Santec, Brittany, France	17a	Low	134134	182182	197197	200200	190190	221221	192192	202202	188188	200200	184188	197197	196196
Santec, Brittany, France	19b	Low	134157	182182	0	200200	190190	221221	192192	202202	188188	200200	184188	197197	196196
Santec, Brittany, France	1a	Low	136136	182182	199199	200200	190190	221221	192192	202202	188188	200200	187187	197197	196196
Santec, Brittany, France	20a	Low	134134	182182	197197	200200	190190	221221	192192	202202	188188	200200	182188	197197	196196
Santec, Brittany, France	20b	Low	132134	182182	199199	200200	190190	221221	192192	202202	188188	200200	188188	197197	194194
Santec, Brittany, France	2b	Low	134164	182182	199199	200200	190190	221221	192192	202202	188188	200200	0	197197	196196
Santec, Brittany, France	3a	Low	134134	181181	199199	200200	190190	221221	192192	202202	188188	200200	185185	197197	196196
Santec, Brittany, France	4a	Low	134134	182182	197197	200200	190190	221221	192192	202202	188188	200200	0	197197	196196
Santec, Brittany, France	5b	Low	134134	182182	199199	200200	190190	221221	192192	202202	188188	200200	0	197197	196196
Santec, Brittany, France	6a	Low	134134	182182	199199	200200	190190	221221	192192	202202	188188	200200	187187	197197	196196
Santec, Brittany, France	6b	Low	157157	182182	199199	0	190190	221221	192192	202202	188188	0	184184	197197	196196
Santec, Brittany, France	7b	Low	134134	182182	199199	200200	190190	221221	192192	202202	188188	200200	184188	197197	196196
Santec, Brittany, France	8a	Low	134134	182182	199199	207207	190190	221221	192192	202202	188188	200200	184188	197197	196196
Santec, Brittany, France	8b	Low	134134	182182	199199	200200	190190	221221	192192	202202	188188	200200	184184	197197	0
Santec, Brittany, France	9a	Low	0	182182	199199	200200	190190	225225	192192	202202	188188	200200	184187	197197	196196
Santec, Brittany, France	9b	Low	134134	182182	197197	207207	190190	221221	192192	202202	188188	200200	188188	197197	194194
Santec, Brittany, France	-10a	High	164164	177177	199199	207207	192192	227227	189189	200202	188188	200200	187187	197197	196196
Santec, Brittany, France	-11a	High	164164	177177	199199	207207	192192	223223	189189	202202	188188	200200	184184	197197	196196
Santec, Brittany, France	-12b	High	164164	177177	199199	207207	192192	227227	189192	200200	188188	200200	187187	197197	196196
Santec, Brittany, France	-13a	High	134163	177182	199199	207207	192192	223223	192192	200200	188188	200200	184184	197197	196196
Santec, Brittany, France	-13b	High	164164	182182	199199	207207	192192	227227	192192	200200	188188	200200	184184	197197	196196

Table GII (cont.). Alleles present in 13 msat loci of *F. spiralis* and *F. vesiculosus* individuals.

Location	Individual	Collected as	Locus												
			L20	**F9**	**F12**	**F21**	**F34**	**F36**	**F42**	**F49**	**F60**	**F17**	**F58**	**F59**	**F72**
Santec, Brittany, France	-14b	High	164164	177177	199199	207207	192192	227227	189189	200200	188188	200200	187187	197197	196196
Santec, Brittany, France	-15a	High	164164	177182	199199	207207	192192	223227	192192	200200	188188	200200	184184	197197	196196
Santec, Brittany, France	-15b	High	164164	177177	199199	207207	192192	227227	189189	200200	188188	200200	187187	197197	196196
Santec, Brittany, France	-16a	High	164164	182182	199199	207207	192192	223223	192192	200200	188188	200200	183187	197197	196196
Santec, Brittany, France	-16b	High	157164	182182	199199	207207	192192	223223	189189	200200	188188	200200	184184	197197	196196
Santec, Brittany, France	-17b	High	157164	182182	199199	207207	192192	221221	189189	200200	188188	200200	184187	197197	196196
Santec, Brittany, France	-17bb	High	0	177177	199199	207207	192192	221227	189192	200202	188188	200200	188188	197197	196196
Santec, Brittany, France	-18a	High	164164	182182	199199	207207	192192	223223	192192	200200	188188	200200	184184	197197	196196
Santec, Brittany, France	-18b	High	164164	177182	199199	207207	192192	223223	189189	200200	188188	200200	184188	197197	196196
Santec, Brittany, France	-19a	High	164164	177182	199199	207207	192192	221221	189189	200200	188188	200200	187187	197197	196196
Santec, Brittany, France	-19b	High	157164	182182	199199	207207	192192	227227	189189	200200	188188	200200	188188	197197	196196
Santec, Brittany, France	-20a	High	164164	182182	199199	207207	192192	221221	189192	200202	188188	200200	187187	197197	196196
Santec, Brittany, France	-20b	High	164164	182182	199199	207207	192192	219221	192192	200200	188188	200200	184184	197197	196196
Perharidy, Brittany, France	550	Unknown	134147	182182	199199	200200	192192	221221	189189	202202	188188	198198	184184	199199	196196
Perharidy, Brittany, France	551	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	200200	184184	199199	196196
Perharidy, Brittany, France	552	Unknown	147147	177182	199199	200200	192192	221221	189189	202202	188188	198198	184184	199199	196196
Perharidy, Brittany, France	553	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	198198	184184	199199	196196
Perharidy, Brittany, France	554	Unknown	0	182182	199199	200200	192192	227227	192192	0	188188	200200	184184	197197	196196
Perharidy, Brittany, France	556	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	198198	184184	199199	196196
Perharidy, Brittany, France	557	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	198198	184184	199199	196196
Perharidy, Brittany, France	558	Unknown	147147	177177	199199	207207	192192	221221	189189	202202	188188	200200	0	199199	195195
Perharidy, Brittany, France	559	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	198198	184184	199199	196196
Perharidy, Brittany, France	560	Unknown	147157	182182	199199	200200	192192	221221	189189	202202	188188	198198	184184	199199	196196
Perharidy, Brittany, France	562	Unknown	171991	177184	199199	200200	192192	221221	189189	202202	188188	198198	0	199199	196196
Perharidy, Brittany, France	563	Unknown	147158	184184	199199	200200	192192	221221	190190	0	188188	200200	0	197197	198198
Perharidy, Brittany, France	564	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	198198	184184	199199	196196
Perharidy, Brittany, France	565	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	198198	184184	199199	196196
Perharidy, Brittany, France	566	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	198198	184184	199199	196196
Perharidy, Brittany, France	567	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	198198	0	199199	196196
Perharidy, Brittany, France	568	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	200200	184184	199199	196196
Perharidy, Brittany, France	569	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	200200	184184	199199	196196

Table GII (cont.). Alleles present in 13 msat loci of *F. spiralis* and *F. vesiculosus* individuals.

Location	Individual	Collected as	Locus												
			L20	**F9**	**F12**	**F21**	**F34**	**F36**	**F42**	**F49**	**F60**	**F17**	**F58**	**F59**	**F72**
Perharidy, Brittany, France	571	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	200200	184191	199199	196196
Perharidy, Brittany, France	572	Unknown	0	177177	199199	0	192192	221221	189189	202202	188188	198198	184184	199199	196196
Perharidy, Brittany, France	573	Unknown	147147	182182	199199	200200	192192	221221	189189	202202	188188	198198	184184	199199	196196
Santec, Brittany, France	11b	*F. vesiculosus*	172172	182182	210210	206212	0	219227	188191	200200	190194	200200	186186	199199	194194
Santec, Brittany, France	12a	*F. vesiculosus*	134169	182182	204204	206212	0	219227	188191	0	188192	200200	186186	201207	194194
Santec, Brittany, France	14b	*F. vesiculosus*	160160	180182	212218	206212	0	219223	191191	200200	192194	200200	186186	197201	194194
Santec, Brittany, France	17b	*F. vesiculosus*	157163	180182	190218	206212	0	0	191191	200202	192192	200200	186186	197199	194194
Santec, Brittany, France	18a	*F. vesiculosus*	157157	182182	210210	206212	0	219223	191191	200200	192192	200200	186186	199199	194194
Santec, Brittany, France	19a	*F. vesiculosus*	147147	182182	210210	206212	0	219223	185191	200200	192194	200200	185185	205205	194194
Santec, Brittany, France	21a	*F. vesiculosus*	169169	182182	208222	206212	0	219227	187191	200200	190192	200200	186186	199199	194194
Santec, Brittany, France	21b	*F. vesiculosus*	134163	180180	208221	206212	0	219227	191191	200200	193193	200200	186186	201201	194194
Santec, Brittany, France	23b	*F. vesiculosus*	157169	180182	190216	206212	0	219223	187191	200200	192192	200200	186186	199201	194194
Santec, Brittany, France	24b	*F. vesiculosus*	157169	182182	196206	206212	0	0	191191	202202	188192	200200	186186	197197	194194
Santec, Brittany, France	26a	*F. vesiculosus*	134134	182182	198202	206212	0	219227	191191	202202	188192	200200	186186	199199	194196
Santec, Brittany, France	28a	*F. vesiculosus*	169169	182182	190218	206212	0	0	187191	200202	192192	200200	186186	197199	194194
Santec, Brittany, France	29b	*F. vesiculosus*	169172	182182	190196	206212	0	0	187191	200202	188192	200200	185185	196201	194194
Santec, Brittany, France	30a	*F. vesiculosus*	170172	182182	190196	206212	0	0	187191	200202	188192	200200	186186	197197	194194
Santec, Brittany, France	31a	*F. vesiculosus*	157157	180182	190190	206212	0	219227	0	200200	192192	200200	186186	199199	194194
Santec, Brittany, France	36b	*F. vesiculosus*	157172	180182	222222	206212	0	219227	187187	200202	192192	200200	185185	200200	194194
Santec, Brittany, France	39a	*F. vesiculosus*	172172	180180	204214	206212	0	0	187187	200202	192192	200200	186186	197205	194194
Santec, Brittany, France	40a	*F. vesiculosus*	161161	182182	216216	206212	0	0	191191	200200	192192	200200	186186	201205	194194
Santec, Brittany, France	40b	*F. vesiculosus*	157161	180182	222227	206212	0	0	192192	200200	192192	200200	186186	197199	194194
Santec, Brittany, France	43a	*F. vesiculosus*	169172	182182	196200	206212	0	219223	192192	200200	188193	200200	186186	199201	194196
Santec, Brittany, France	44b	*F. vesiculosus*	160160	180180	190190	206212	0	223223	192192	200200	193193	200200	186186	199201	194194
Santec, Brittany, France	45b	*F. vesiculosus*	172172	180180	196210	207212	0	219223	188192	202202	192192	200200	186186	199205	181194
Santec, Brittany, France	4b	*F. vesiculosus*	147170	182182	196212	207212	0	219223	192192	202202	192192	200200	186186	197197	194194
Santec, Brittany, France	7a	*F. vesiculosus*	161172	182182	214227	206212	0	0	188192	200200	192192	200200	186186	199199	194194