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Evolution of mating systems and their implication in the processes of speciation and hybridization in brown algae of the genus *Fucus*

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Evolution of mating systems and their implication in the processes of speciation and hybridization in brown algae of the genus *Fucus*

Summary

In the genus *Fucus* the character dioecy/hermaphroditism has evolved several times and hybridization is possible between taxa with contrasting mating systems, making it an excellent model to study evolution of mating systems at both macro and micro-evolutionary scales.

A phylogenetic approach based on intergenic chloroplast sequences showed that, like in higher plants, dioecy evolved from ancestral hermaphroditism in algae. However, relationships between taxa *F. vesiculosus*, *F. spiralis* and *F. ceranoides* are still unresolved, questioning their species status.

Using markers of gene flow we confirmed that the three species were reproductively isolated although not totally.

In order to study the importance of barriers to gene flow, we focused our analysis at the shore scale which is the transition scale between *F. vesiculosus* and *F. spiralis*. Different approaches, including population genetics and population biology have been used. As expected according to ecological models of reproductive systems evolution, the hermaphroditic species shows a very low sperm/egg ratio while resource allocation towards male function is observed in the dioecious species. Our results show that hybridisation events are spatially limited by low dispersal capabilities and a high selfing rate in *F. spiralis*. They are mainly due to of *F. vesiculosus*' sperm fertilizing *F. spiralis*' eggs.

These results provide new evidences for the importance of mating system in the process of speciation.

Key-words

Fucus, mating systems, speciation, hybridization, phylogeny, population genetics, sexual allocation, ecological divergence, Portugal, Brittany

Evolução de sistemas de reprodução e a sua implicação nos processos de especiação e hibridação em algas castanhas do género *Fucus*

Resumo

No género *Fucus*, o carácter dioicismo/hermafroditismo evoluiu várias vezes e a hibridação é possível entre *taxa* com sistemas de reprodução contrastantes, fazendo com que seja um modelo excelente para estudar a evolução de sistemas de reprodução tanto em escalas macro como micro-evolutivas.

Uma abordagem de filogenia baseada em sequências intergénicas do cloroplasto mostrou que, em algas como em plantas superiores, a dioicia evoluiu a partir de hermafroditismo ancestral. Contudo, as relações entre as espécies *F. vesiculosus*, *F. spiralis* e *F. ceranoides* ainda não estão resolvidas, o que coloca questões relativamente à sua posição como espécies. Utilizando marcadores do fluxo genético, confirmámos que as três espécies estão reprodutivamente isoladas embora não totalmente.

Para estudar a importância de barreiras ao fluxo genético, focámos a nossa análise na escala da região intertidal, que é a escala de transição entre *F. vesiculosus* e *F. spiralis*. Várias técnicas foram utilizadas, incluindo genética de populações e biologia de populações. Como esperado segundo os modelos ecológicos da evolução de sistemas reprodutivos, a espécie hermafrodita mostra uma proporção de espermatozóides/óvulos muito baixa enquanto que maior alocação de recursos na função masculina é observada nas espécies dioicas. Os nossos resultados mostram que os eventos de hibridação são limitados pelo espaço devido a capacidades de dispersão baixas e uma elevada autofecundação em *F. spiralis*. Estes eventos são principalmente devidos a uma fertilizacao dos óvulos de *F. spiralis* pelos espermatozóides de *F. vesiculosus.*

Estes resultados fornecem novas evidências sobre a importância dos sistemas de reprodução no processo de especiação.

Palavras chave

Fucus, sistema de reprodução, especiaçao, hibridação, filogenia, genética de populações, alocação sexual, divergência ecológica, Portugal, Bretanha francesa

Evolution des systèmes de reproduction et leur implication dans les processus de spéciation et hybridation chez les algues brunes du genre *Fucus*

Resumé

Chez les algues brunes du genre *Fucus*, le caractère dioécie/hermaphrodisme a évolué plusieurs fois et l'hybridation est possible entre taxa possédant des systèmes de reproduction contrastés. Ces singularités en font un excellent modèle pour étudier l'évolution des systèmes de reproduction aussi bien à l'échelle macro que micro-évolutive.

Une approche phylogénétique basée sur l'analyse de séquences chloroplastiques démontre que, comme chez les plantes supérieures, l'hermaphrodisme est ancestral chez ces algues. Cependant, les taxa *F. vesiculosus, F. spiralis* et *F. ceranoides* sont compris dans un râteau irrésolu, mettant en question leur statut d'espèce.

Par l'utilisation de marqueurs du flux génique nous avons démontré que les trois espèces étaient bien isolées reproductivement quoique incomplètement.

Afin d'étudier l'importance de la barrière aux flux géniques, nous avons concentré notre analyse à l'échelle d'un estran, zone de transition entre *F. vesiculosus* et *F. spiralis*. Différentes approches, alliant génétique et biologie des populations ont été utilisées. Comme attendu selon les modèles écologiques de l'évolution des systèmes de reproduction, l'espèce hermaphrodite montre un très faible ratio sperme/ovule tandis qu'une réallocation des ressources vers la fonction mâle est constatée chez l'espèce dioïque. Nos résultats démontrent que l'hybridation est fortement limitée spatialement par de faibles capacités de dispersion et un fort taux d'autofécondation chez *F. spiralis*. Elle est due au sperme *F. vesiculosus* fécondant les ovules de *F. spiralis.*

Ces résultats apportent de nouvelles preuves de l'importance des régimes de reproduction lors du processus de spéciation.

Mots clé

Fucus, système de reproduction, spéciation, hybridation, phylogénie, génétique des populations, allocation aux fonctions sexuelles, divergence écologique, Portugal, Bretagne

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1 GLOSSARY

Antheridium (antheridia): the male reproductive organ (gametangium) that produces male gametes in oogamous sexual reproduction.

Blade, frond or lamina: part of a thallus that is erect and more or less flattened or leaf-like.

Conceptacle: in the Fucaeae (Phaeophyceae) and Corallinaceae (Rhodophyceae), an invagination or cavity (either sunken or within a raised dome) having one or more openings to the thallus surface and bearing reproductive organs

Gametophyte: individual stage producing haploid (n) gametes

Heterokontae: lineage of eukaryotic organisms that have heterokont or unequal flagella, also Stramenopiles.

Heterotrichous: a filamentous system composed of prostrate and erect filaments.

Oogonium (oogonia): the female reproductive organ (gametangium) that produces one or more ova or eggs.

Parenchymatous: a parenchymatous tissue is composed of thin-walled, undifferentiated cells, resulting from the mitotic divisions of a meristematic tissue; most often functioning in photosynthesis or storage

Receptacle: in the Fucales (Phaeophyta) a fertile specialized area of the thallus on which gametangia are produced.

Sporophyte: individual stage producing spores

Stipe: a basal stalk-like portion of a thallus or any stem-like portion of a thallus, either cylindrical or flattened

Thallus (thalli): a macroalgal body, with no differentiation into true roots, stems or leaves.

INTRODUCTION

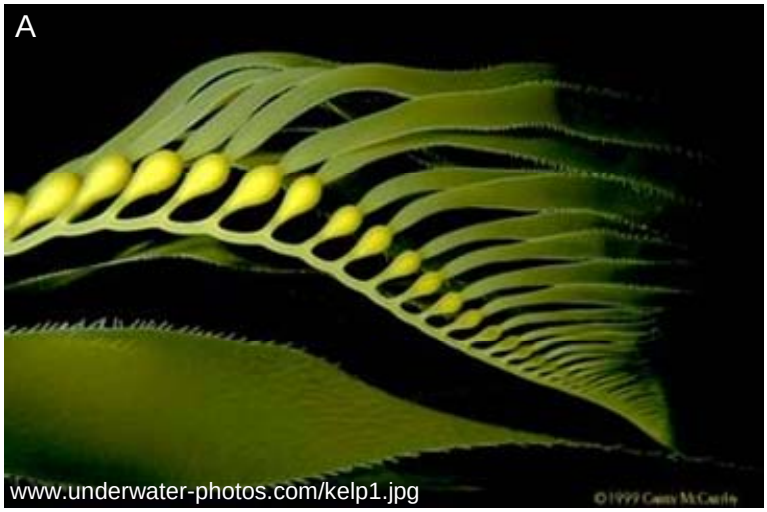
2 INTRODUCTION

Life history traits like lifespan, dispersal capacity or life cycle have a great evolutionary importance since, by influencing the mating systems, they can act on population genetic structure and local adaptation possibilities. As a consequence, they are subject to selection and are likely to evolve. For more than a century, these questions of evolutionary biology have been addressed in plants (Silvertown & Harper, 1997; Vuorisalo & Mutikainen, 2001) and in animals (Roff, 2001; Stearns, 1992). On the contrary, relatively few studies have been done on algae, despite their high variability in life history traits, which has long been described at different taxonomic levels (Fritsch, 1945; Scagel et al., 1982). Algae are particularly interesting models to study life cycle evolution (Feldman, 1972; Mable & Otto, 1998; Valero et al., 1992) and more specifically questions about the evolution of diploidy and breeding system. The general purpose of this thesis focuses on the question of the evolution of reproductive systems using macro-algae of the class Phaeophyceae as a study model.

Life cycle evolution

The term « alga » refers to a heterogeneous group of generally autotrophic and aquatic organisms from different evolutionary lineages (De Reviers, 2002; 2003; Lecointre & Le Guyader, 2001). Phaeophyceae belong to the heterokont lineage (with Diatoms and oomycetes) very distant from green and red algae lineages (Baldauf, 2003). Phaeophyceae are multicellular marine organisms exhibiting diverse morphologies from filamentous branched thalli (heterotrichous) to complex parenchymatous thalli with conductive tissues. The colour of brown algae is due to the presence of fucoxanthin, a xanthophyll pigment. In addition to cellulose, cell walls are made of other polysaccharides like alginates or fucans, both molecules that are used in the food-processing and pharmaceutical industries. Thus, several species of brown algae present economical interest and are industrially exploited (crops and harvesting). In brown algae, Clayton (1988) distinguished three kinds of life cycles:

- The **heteromorphic haplo-diploid cycle**, defined by the alternation of haploid (gametophytes) and diploid (sporophytes) individuals; both stages presenting different morphologies. In Laminariales, for example, heteromorphy is extreme since the gametophyte is microscopic whereas the sporophyte may reach several tens of meters (e.g., *Macrocystis pyrifera*, picture 1)



Pictures 1A and 1B: Sporophyte of the giant kelp *Macrocystis pyrifera*. A: detailed view of the sporophyte bladders. B: overview of a kelp forest, in this species, sporophytes can reach 70 meters in length, whereas the gametophyte is microscopic.

- The isomorphic haplo-diploid cycle, in which stages, haploid and diploid, do not present any pronounced morphological difference, like species of the genus *Ectocarpus* (picture 2).

Source : Rubén Chapela Orri,
Station Biologique de Roscoff

Picture 2: *Ectocarpus fasciculatus*, filamentous alga, few centimetres long.
The sporophyte and gametophyte are similar.

- The diploid cycle, in which the diploid sporophyte is the only free stage; gametophytic stage being reduced to gamete production. These cycles are mainly observed in perennial intertidal algae like Fucales (picture 3)

Fucus spiralis

Fucus vesiculosus

Pictures 3_A and 3_B: The two species of Fucales with a diplontic cycle, very frequent on the intertidal rocky shores in Europe, which will be studied along this thesis for their reproductive systems: *Fucus spiralis* (hermaphroditic) and *F. vesiculosus* (dioecious).

Numerous hypotheses have been suggested concerning the evolution of life cycles, particularly to explain the persistence of both, haploid and diploid, stages (Coelho et al., 2007; Mable & Otto, 1998; Valero et al., 1992). In the case of a heteromorphic cycle, the classical explanation is the adaptation of each stage to different environmental conditions (Clayton, 1988; Lubchenco & Cubit, 1980; Stebbins & Hill, 1978). The model of Hughes & Otto (1999) shows that this alternation can be maintained if the two stages use different resources: for example if one stage is resistant to grazers (crustose form) whereas the other stage has a competitive advantage for growth (erect form).

Bell (1997) proposed to test the different hypotheses about life cycle evolution in Phaeophyceae which present, like land plants, a trend for larger diploid sporophytes concomitant with smaller haploid gametophytes. To explain this trend, he came out with an ancient hypothesis first proposed by Bower (1908), which leans on the functional differences between gametes (fusion/fertilization) and spores (dispersion). Heteromorphism could thus be explained by the selection in gametophytes of small size, maximizing gamete encounters by the vicinity of males and females. On the contrary, the large, erect form of the sporophyte is supposed to be selected to enhance spore dispersion and colonization. Finally, Bell (1997) showed that in the Phaeophyceae, the classical

sexual cycle (with the haploid stage specialized as a gametophyte and the diploid stage specialized as a sporophyte) is not the general rule. Indeed, some species are characterized by the alternation of macrothallus (sporophyte) and microthallus (gametophyte) in an asexual cycle. Thus, he concludes that, in some groups, genetic theories on the advantages of diploidy did not fit in with the alternation of the different stages.

Several genetic models of life cycle evolution have been proposed. Perrot et al. (1991) lean on a genetic load model to predict that, given the protection against recessive deleterious mutations it confers, diploidy should be favoured and lead to an increased number of segregating mutations in populations. Indeed, in diploids, selection against deleterious alleles is less efficient because they are masked. Considering the evolution of a modifier gene controlling life cycle (Bokn et al., 2002; Otto & Goldstein 1992; Otto & Marks, 1996) Otto showed that two effects influence the evolution of cycles: diploidy is advantageous at short-term (masked mutations), whereas haploidy is more advantageous at long-term, due to greater efficiency of selection in haploids. On the one hand, when recombination is low (asexual reproduction or inbreeding), modifier alleles coding for a longer haploid phase will be advantageous because they can be beneficial as a long term effect. On the other hand, when recombination is important (sexual reproduction and out-crossing), the benefits of the purge occurring during the haploid phase are distributed among all modifier alleles by recombination. As a consequence, haploidy will be favoured by low recombination conditions whereas diploidy will be favoured with high recombination. Thus, these models make two predictions: 1) In populations where recombination is important, transition from haploidy to diploidy is expected. 2) Evolution (or maintenance) of haploidy should be associated with low recombinant breeding systems, like clonality or inbreeding (Otto & Marks, 1996).

Bell (1997) proposed to test these two predictions in brown algae. Firstly, to test the former, he used the phylogenetic data published by Tan & Druehl (1993) and base on ribosomal RNA 18S. Unfortunately, at that time, data were not sufficient to assign with confidence the ancestral state of life cycle in this group. This question deserves now to be readdressed in the light of new results obtained for Phaeophyceae and reviewed by De Reviers et al. (in press).

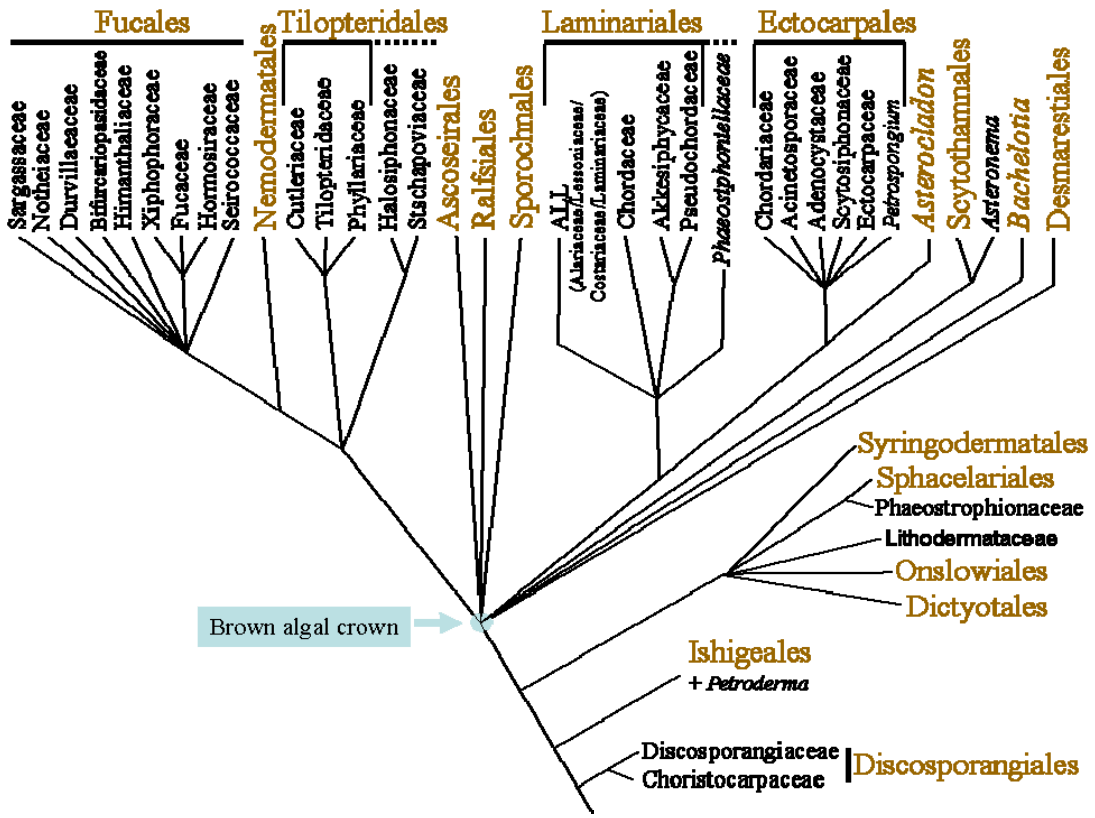


Figure 1: Synthetic tree of the evolution of Phaeophyceae, built from results of different studies. These results were obtained mainly with chloroplastic markers (psaA, RbcL and RbcL/S spacer (ex: Cho & Boo, 2006; Cho et al., 2006)) from De Reviers et al. (in press).

Contrary to all previous phylogenetic hypotheses, data now clearly show the ancestrality of the isomorphic haplo-diploid cycle in this group (De Reviers et al, in press). From this ancestral state, numerous groups have evolved toward a predominant diploid phase, or even an entirely diploid cycle; the remaining groups kept an isomorphic cycle and some of them, like the Scytosiphonaceae, have evolved toward a predominant haploid phase.

Secondly, in order to test if inbreeding (or clonality) is more frequent in groups with dominant haploid stage, Bell (1997) compared monoecious and dioecious species, his hypothesis being that monoecious species were more inbred. However, he found that monoecy was not more often associated with haploidy than with diploidy. Moreover, he looked at the ability of species to produce sporophytes by parthenogenesis in order to have an estimation of the asexual reproduction. Once again, he showed no correlation

with haploidy and therefore he rejected the genetic theories of the advantage of diploidy. Nevertheless, his approach can be criticized because neither monoecy nor the ability to produce parthenosporophytes are direct measures of the reproductive system. To which extent are life cycles and reproductive system linked together? Data on population genetic structure are necessary to estimate correctly reproductive systems. This kind of study is still not very developed in algae. Indeed, before the review of De Soza & Lindstrom published in 1999, only three papers were published on the genetic variability of brown algae populations (Lu & Williams, 1994; Neefus et al., 1993; Williams & Di Fiori, 1996). However, more and more data are becoming available especially for Fucales and Laminariales (table 1).

Table 1: Examples of different estimations of population genetic structure in brown algae.

Species	*Reproductive System*	*Author*	*Marker*	*A*	*He*	F_{IS}
Halidriys dioica	dioecious	Lu & Williams 1994	isozymes	1.8	0.17 – 0.23	0.57 – 0.64
Laminaria digitata	dioecious	Billot et al., 2003	msat	3.4 – 6.7	0.47 – 0.70	-0.01 ^{ns} - 0.19*
Undaria pinnatifida	dioecious	Voisin et al en prep	msat	2.3 – 3.02	0.30 – 0.44	0.02 ^{ns} - 0.69*
Fucus serratus	dioecious	Coyer et al., in press	msat	8.7	0.5	0.08
F. vesiculosus	dioecious	Engel et al., 2005	msat	7.8	0.67	0.16*-0.25*
-	dioecious	Perrin et al., 2007	msat	9.5	0.75	0.01 ^{ns} -0.21*
-	dioecious	Wallace et al., 2004	msat	6.7	0.57	0.31*
-	dioecious	Tatarenkov et al., 2007	msat	4.9	0.57	0.07^{ns}
Postelsia palmaeformis	dioecious	Kusumo et al., 2006	msat		0.22 – 0.50	0.28 – 0.68[1]
S. compressa	hermaphroditic	Williams & Di Fiori, 1996	isozymes	1 - 2	0 – 0.88	0.03 – 0.65
F. distichus	hermaphroditic	Coleman & Brawley, 2005b	msat	4.75 - 7.5	0.53 – 0.57	0.45*-0.63*
F. evanescens	hermaphroditic	Coyer et al., in press	msat	2.9	0.12	0.77
F. spiralis	hermaphroditic	Perrin et al., 2007	msat	5.8	0.43	-0.58* - 0.78*
-	hermaphroditic	Coleman & Brawley, 2005a	msat		0.33 - 0.58	0.24*- 0.48*
-	hermaphroditic	Engel et al., 2005	msat	2.6	0.21	0.89* - 1.00*
-	hermaphroditic	Wallace et al., 2004	msat	8	0.39	0.60*

A: Mean number of alleles per locus, He: expected heterozygosity when populations are at Hardy Weinberg equilibrium; F_{IS}: Estimator of heterozygote deficit (if F_{IS} >0, the population presents a heterozygote deficit); *significant value, ns non significant value, msat: microsatellite marker; 1 F_{IS} values are calculated according to the formula F_{IS} = 1-(Ho/He)

Although hermaphroditic species generally present higher F_{IS} values than dioecious species, several exceptions are noteworthy (table 1) such as for example, the high F_{IS} values observed in *Halidrys dioica* compared with those observed in the same region for the hermaphroditic species *Silvetia compressa*. Moreover, F_{IS} values estimated

for dioecious species are often significantly positive, which suggests repeated inbreeding events. This table also reveals great variations in the estimations of mating system within species, suggesting that mating system may vary between populations within species. In *F. vesiculosus* and *Undaria pinnatifida* for example, shifting towards asexual reproduction seems to occur in populations submitted to particular environmental conditions (brackish water for *F. vesiculosus* (Tatarenkov et al., 2005) and stagnant water for *U. pinnatifida* (Voisin, pers. comm.). What is the link between reproductive system and mating system? Although in plants, an important body of literature exists, the question of reproductive system evolution in algae has been largely ignored. This is what will be addressed in the following part of this introduction.

Mating system evolution

From the great diversity of their reproductive systems, from hermaphroditism, co-sexuality where each individual carries both male and female organs, to dioecy, uni-sexuality where individuals possess only one type of sexual organ, plants have always been a first choice model to study reproductive system evolution (for review, see: Barrett & Harder, 1996; Charlesworth, 2006; Cheptou & Schoen, 2007; Geber et al, 1999; Goodwillie et al., 2005). Hermaphroditism confers many advantages compared with dioecy. From an ecological standpoint, selfing is possible when individuals are isolated (Barrett & Harder, 1996) and from a genetic standpoint, a hermaphrodite transmits its whole genome to its offspring, via male and female functions, theoretically doubling its fitness compared with unisexual individuals (Fisher, 1941). Although this reproductive system is widely spread (Vogler & Kalisz, 2001), dioecy seems to have evolved independently in different taxa (Barrett & Case, 2006; Bawa & Beach, 1981 ; Desfeux et al, 1996) and theoretical, empirical and phylogenetic studies converge to suggest co-sexuality as the ancestral state and uni-sexuality as the derived state in angiosperms (Gebert et al. 1999). Theoretical studies show that three major factors influence reproductive system evolution: 1) the short-term advantage of selfing, 2) the importance of inbreeding depression, 3) autogamous individual contribution to gametic pool (Charlesworth & Charlesworth, 1990; Holsinger, 1988; Lande & Schemske, 1985). One major hypothesis explaining the evolution towards separate sexes assumes that dioecy

could have evolved from hermaphroditism to avoid inbreeding depression effects, particularly in habitats where competition is important. Indeed, in case of limited resources, it may be more advantageous to have only one type of sexual organ (Charnov, 1982, Figure 2)

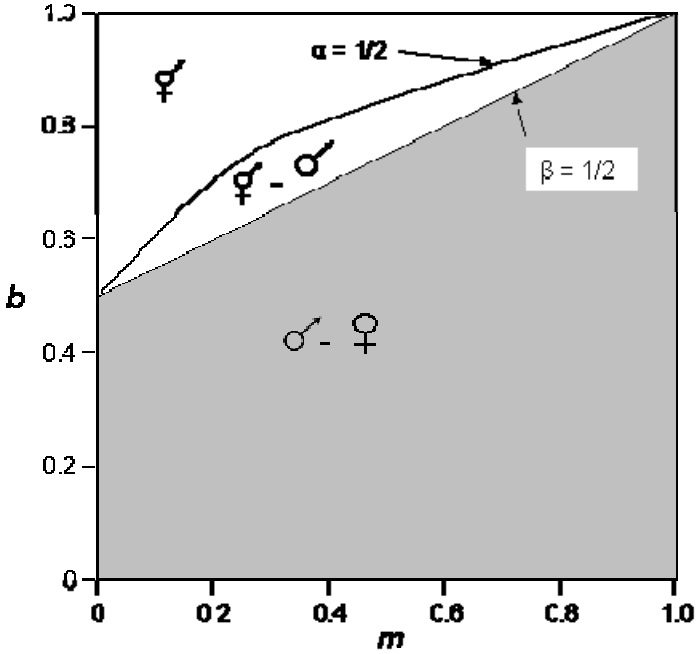


Figure 2 : From Charnov (1979) Why be an hermaphrodite? Male fitness is given by the relation $\alpha = br^m$, female fitness is given by the relation $\beta = (1-r)b$, r being the resource allocated to male function and m the fertility gain associated to resource allocation.

Although numerous genera are composed by hermaphroditic and dioecious species in algae (genera *Oeogonium*, *Porphyra*, *Phyllophora Bryopsis* and *Fucus* for example (Brawley & Jonhson, 1992; see also bold & Wynne, 1985; Hawkes, 1990)), comparatively with higher plants, relatively few studies have explored the question of reproductive system evolution in marine algae. However, this question has been studied in Fucales (Phaeophyceae), where the character dioecy/hermaphroditism seems to have evolved several times during the evolutionary history of the taxon (Serrão, 1999; Figure 3).

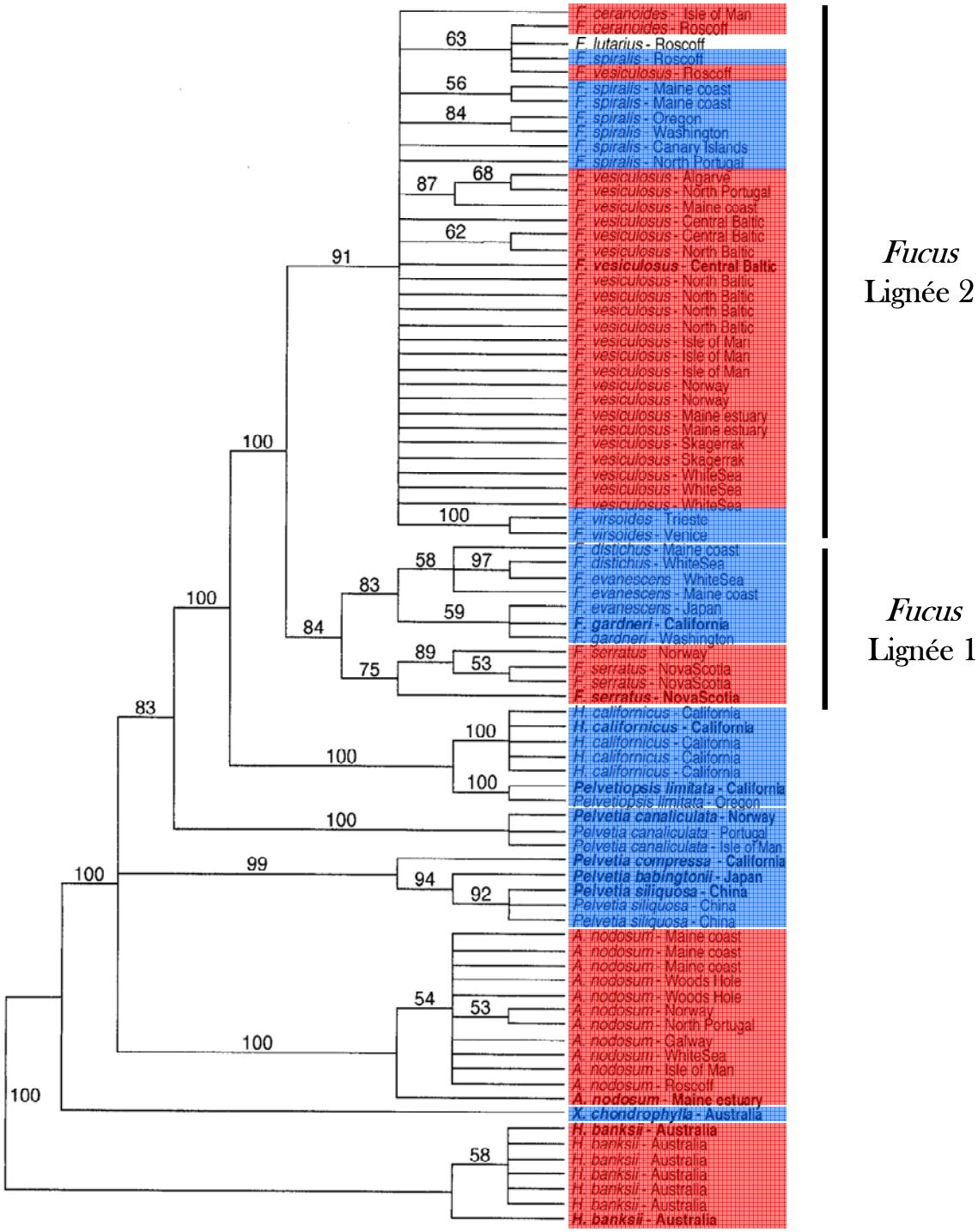


Figure 3: Consensus tree obtained from ITS (Internal Transcribed Spacer) sequences, presenting the evolution of reproductive systems in the Fucaceae family. Bootstrap values are indicated. Species highlighted in blue are hermaphroditic while species highlighted in red are dioecious (from Serrão et al., 1999)

The scattering position of hermaphroditic and dioecious species in the tree (Figure 3) suggests that the reproductive system evolved several times, probably by simple mechanisms (Serrão et al., 1999). This study shows that the ancestral state (hermaphroditic or dioecious?) of the reproductive system is still unresolved in the Fucales and that this group presents a particularly interesting situation to explore the relative importance of ecological and genetic constraints on the reproductive system evolution. However, it appears also that the nuclear ribosomal marker used by Serrão et al. (1999) was not informative enough to resolve phylogenetic relationships between closely related species with contrasting reproductive systems, like the hermaphroditic *F. spiralis* and the dioecious *F. vesiculosus* and *F. serratus* (Figure 3). The development of new genetic markers appears to be a necessary step to the study of the evolution of reproductive systems in this group.

A second argument can be put forward to highlight the interest of the genus *Fucus* for the reproductive system evolution study. In this genus, hybridization between species has long been suspected, particularly between species with contrasting reproductive systems. Indeed, putative hybrids of Fucus have been observed in the field since the beginning of last century (Burrows & Lodge, 1953; Sauvageau, 1909; Stomps, 1911). Since the last five years, the development of molecular tools allowed to genetically confirm this hybridization between the dioecious *F. serratus* and the hermaphroditic *F. evanescens* (Coyer et al., in press ; 2002) on one hand and between the dioecious *F. vesiculosus* and the hermaphroditic *F. spiralis* (Engel et al, 2005 ; Wallace et al, 2004) on the other hand. In addition to the phylogenetic approach, the two sympatric species *F. spiralis* and *F. vesiculosus* appeared thus as excellent models to study the variations of reproductive systems in a context of hybrid zones. In hybrid zones, the population homogenising effect of hybridization is highly counter-balanced by natural selection maintaining genome integrity (Barton & Hewitt, 1989). When first generation hybrids (F1) are fertile, they can potentially back-cross with one of the parental species. This species will thus integrate to its own genome a part of the second species genome. It is to say that this species is introgressed by the second one. Introgression can be symmetrical or not. How does reproductive system evolve in such a system? Is hermaphroditism maintained to limit hybridization? These are the question that we wanted to address during this thesis in species of the genus *Fucus*.

The biological Model

The genus *Fucus* (Fucales, Phaeophyceae) is one of the six genera composing the Fucaceae family. Species of genus *Fucus* are widely distributed along the Northern Atlantic rocky shores (Figure 4). Some species are found on the Pacific coasts and one species in the Adriatic Sea. They dominate the intertidal area where their ecological role is essential, being used as habitat, shelter or food by numerous species.

Some *Fucus* species are restricted to brackish water of estuaries like *F. ceranoides* (Figure 5B, 5F). The two species *F. vesiculosus* and *F. spiralis* are found in sympatry along European shores, although they do not present exactly the same ecological distribution. Indeed, *F. spiralis* occupies the upper intertidal zone whereas *F. vesiculosus* is observed lower in the intertidal area, although both have overlapping distribution zones.

Figure 4 : worldwide distribution of the species of the genus *Fucus*

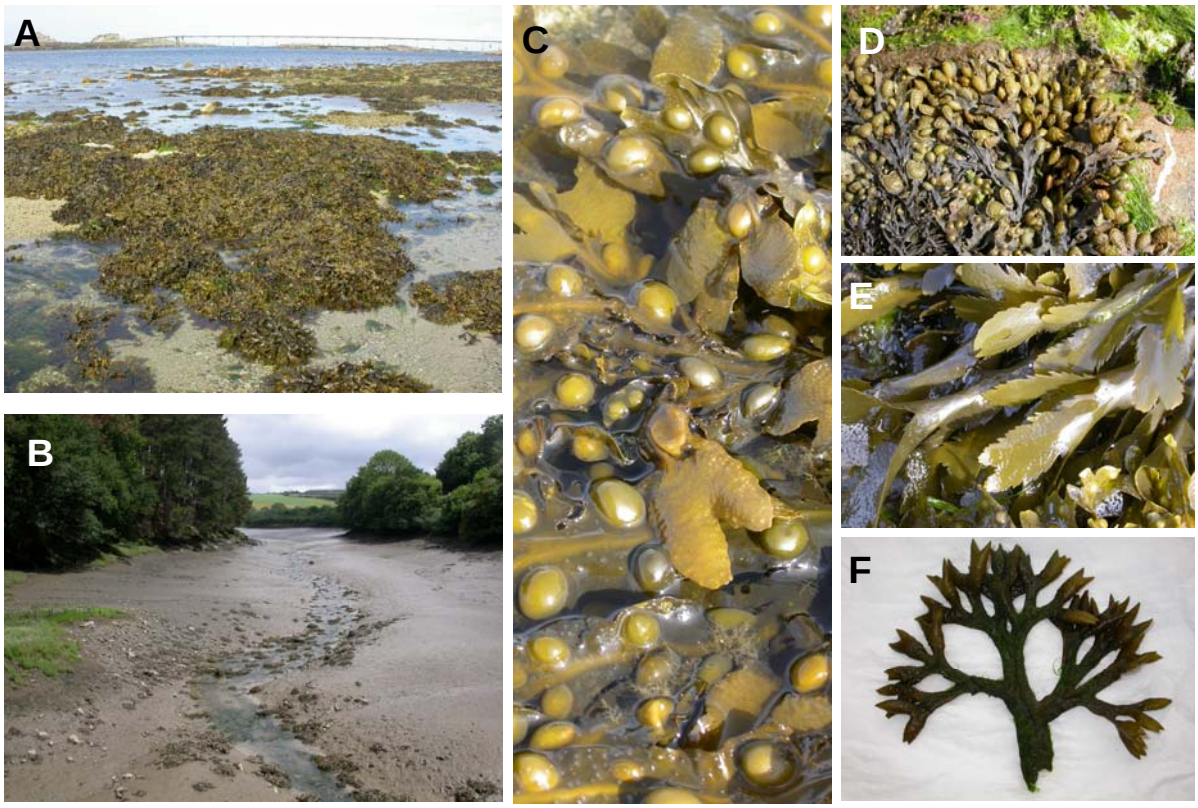


Figure 5 $_{A}$: *Fucus* populations of open coast, $_{B}$: in estuary. $_{C}$: *F. vesiculosus* with bladders, $_{D}$: *F. spiralis*, $_{E}$: *F. serratus*, $_{F}$: *F. ceranoides* restricted to estuaries.

The *Fucus* cycle is diplontic. Diploid individuals present bulging fertile zones called receptacles (Fig. 6). These receptacles can be either female (Fig. 6_{CF}), male (Fig. 6_{CM}) or hermaphroditic (Fig. 6 $_{CH}$) depending on whether the species is dioecious or hermaphroditic. Female and hermaphroditic conceptacles contain oogonia made of eight eggs (female gametes), whereas male and hermaphroditic conceptacles contain antheridia releasing at maturity 64 antherozoids (male gametes). Fertilization is external in all fucoids: during reproductive events, gametes are released from conceptacles by ostioles, visible from the receptacle surface (Fig. 6). The spawning of both types of gametes is synchronised and depends on environmental conditions (Serrão et al, 1996) resulting in a particularly high fertilizing rate (Pearson & Serrão, 2006).

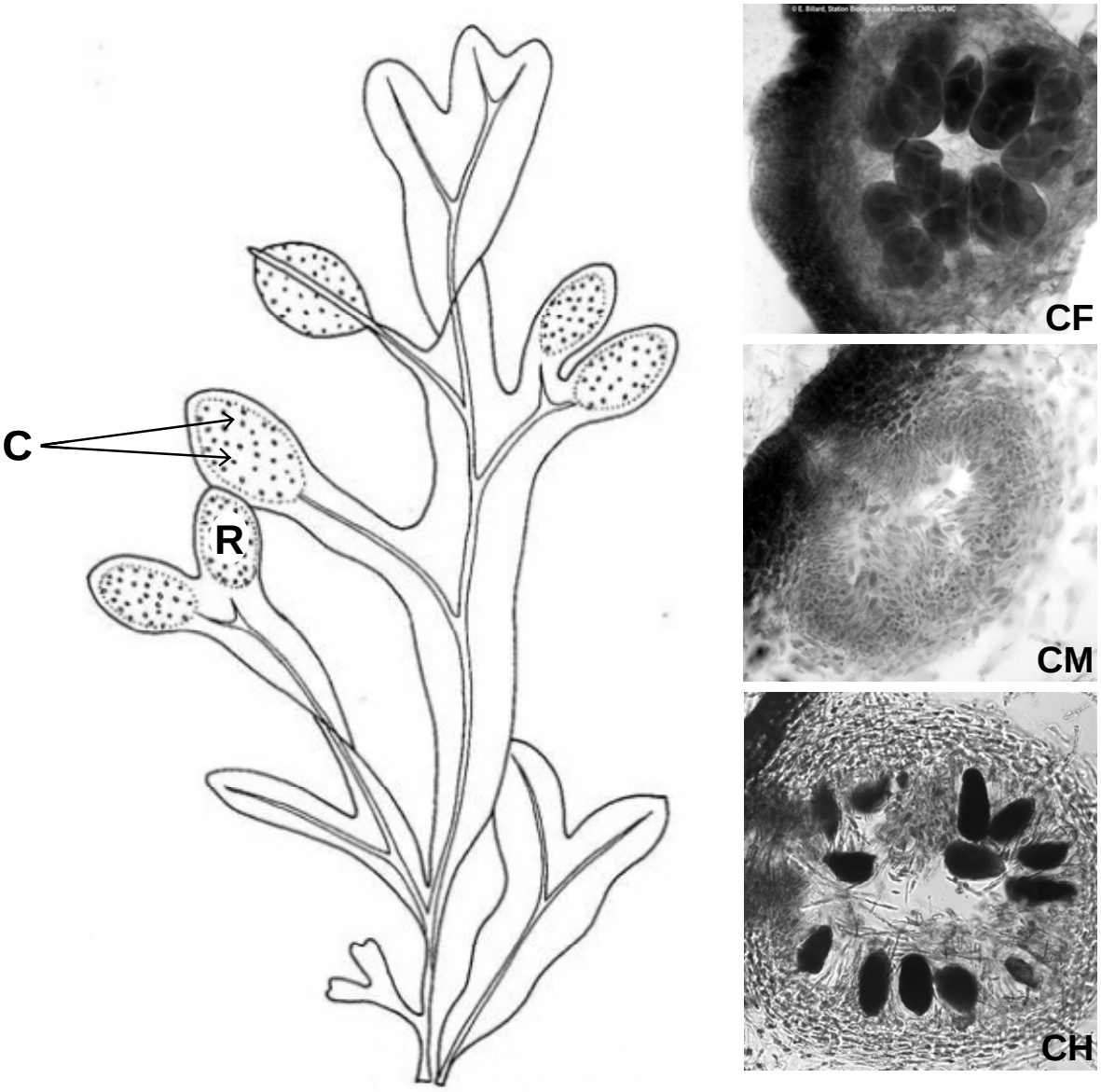


Figure 6: General morphology of *Fucus* (from Coppejans), R: Receptacle, C: conceptacle, with ostiole which can be seen at the receptacle surface. CF: female conceptacle with oogonia, CM: male conceptacle with antheridia, CH: hermaphroditic conceptacle with oogonia and antheridia.

Thesis goals

This present document is divided in four chapters presented as article and ordered according to the different objectives of this work.

1) The first chapter focuses on the question of reproductive system evolution in Fucales in order to test if, like in higher plants, hermaphroditism is the ancestral state in brown algae. This question is addressed with a phylogenetic analysis based on cytoplasmic markers developed during this thesis (Annex 1).

2) The second chapter questions the species status within the complex *F. vesiculosus*/ *F. spiralis*/ *F. ceranoides*. Markers of gene flow have been used to assess the importance of the genetic barriers between taxa of this complex. Indeed, it has still not been possible to distinguish these taxa with phylogenetic methods. It thus seems important to quantify the importance of genetic isolation between these groups by gene flow markers. Additionally, inconstancy of reproductive system having been reported in *F. ceranoides* (Hamel, 1939), it seems necessary to check if these individuals are not hybrids between *F. vesiculosus* and *F. spiralis*.

3) The third chapter aims at comparing the pattern of resource allocation to male and female function between dioecious and hermaphroditic taxa. Are the expectations of the ecological model of resource allocation verified in these brown algal species?

4) In the fourth chapter, we will analyse at the scale of the transition zone of the shore, what are the mating systems in the dioecious and hermaphroditic species and what is their influence on the genetic exchanges between species

In conclusion, a synthesis of the main results obtained during this thesis will be given, highlighting the interest of combining different approaches for this study.

Additonal aspects of this study are given in appendices.

Appendix 1: Engel CR, Billard E., Voisin M & Viard F. (in press) Conservation and polymorphism of mitochondrial intergenic sequences in brown algae. Journal of Phycology

Appendix 2: Amplification protocol of microsatellite loci and chloroplastic markers and restriction protocol of Ase1 and Ssp1 enzymes.

Appendix 3: Sequences obtained for chloroplastic markers thiG-ycf54, psbX-ycf66 and Rubisco.

Appendix 4: Allelic frequencies of individuals sampled along transects.

Appendix 5: Examples of crosses performed during the thesis

ARTICLE 1

Evolutionary history of mating system among the Fucaceae (Phaeophyceae) inferred from a phylogenetic study based on intergenic chloroplast sequences

3 EVOLUTIONARY HISTORY OF MATING SYSTEMS AMONG THE FUCACEAE (PHAEOPHYCEAE) INFERRED FROM A PHYLOGENETIC STUDY BASED ON INTERGENIC CHLOROPLAST SEQUENCES

Billard E. [1,2], Valero M[2], Pearson G. [1], Caetano S. [1], and. Serrão, E. [1], Evolutionary history of mating systems among the Fucaceae (Phaeophyceae) inferred from a phylogenetic study based on intergenic chloroplast sequences. To be submitted in *Journal of Phycology*

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3.1 Abstract

In the family Fucaceae, the characters hermaphroditism and dioecy are distributed among the different genera, suggesting that several independent switches of the mating system occurred during the evolution of this family. Within the genus Fucus in particular, two lineages have been identified on the basis of nuclear and mitochondrial DNA, both of them composed by very closely related hermaphroditic and dioecious species. Until now the ancestral state of mating system in this genus remains unclarified. On the basis of the complete chloroplast genome of *F. vesiculosus*, we selected and analysed three regions in order to reassess the phylogenetic relationships in this family with special regard to the mating system. The level of polymorphism shown by the chloroplast markers makes them more useful at the intergeneric level than at the intra-generic level, although the rubisco-spacer was shown to be discriminant between species in the Fucus genus. Our new dataset now provides good statistical support to conclude that *Ascophyllum* and *Silvetia* are a monophyletic clade. In addition, hermaphroditic *Pelvetia* being the only sister group of the cluster composed by the hermaphroditic genera *Hesperophycus*/*Pelvetiopsis* and the genus *Fucus*, now supports the

hypothesis of dioecy evolving from ancestral hermaphroditism independently in several branches of Fucaceae rather than some reversal of dioecy to hermaphroditism. Our results also support the Pacific origin of Fucaceae and several switches from the Pacific to the Atlantic. The question of where did dioecy evolve still remains to be elucidated, although all current dioecious taxa are Atlantic endemics, suggesting better adaptive value of dioecy in Atlantic intertidal coastlines.

Keywords
Brown algae, chloroplast DNA, mating system, evolution, Evolution, phylogeny, *Fucus*

Abrevations:
cpDNA, chloroplastic DNA
mtDNA, mitochondrial DNA
ML analysis, Maximum-llikelihood analysis
MP analysis, Maximum-parsimony analysis
ITS, internal transcribed spacer
Rbc, Rubisco

3.2 Introduction

Mating system evolution in marine algae is poorly understood despite their high variability. The family Fucaceae is an excellent model for studying evolution of mating systems at a macro-evolutionary scale because the character dioecy/hermaphroditism has undergone multiple switches (Serrão et al. 1999). The scattered phylogenetic positions of hermaphroditic and dioecious species along the evolutionary history of the Fucaceae suggests that either mating system has evolved independently several times, possibly by relatively simple mechanisms (Serrão et al. 1999) as in the flowering plant genus *Silene* (Desfeux et al., 1996), *Wurmbea* (Barrett and Case 2006), and in angiosperms generally (for review see Charlesworth 2002). However, while in land plants, based on modelling, empirical and phylogenetic studies, dioecy generally appears to be the derived state arising from cosexual ancestors (Charlesworth 1999), in the Fucaceae, partly due to the lack of discriminating markers, the ancestral state is still questionable. Yet, in the genus *Fucus*, the occurrence of relics of male function in hermaphrodites and females of the sister species *F. vesiculosus* and *F. spiralis*, suggests that hermaphroditism is the ancestral state (Billard et al. 2005b).

The family Fucaceae appears to have had its origin in the North Pacific Ocean, presumably resulting from a transequatorial crossing of an ancestor of Australasian origin, as suggested by several independent phylogenies (Serrão et al. 1999, Draisma et al. 2001, Cho et al. 2006). The closest living relatives of the Fucaceae are thus the two Australasian families *Hormosiraceae* (currently including only the dioecious species *Hormosira banksii*) and newly created (Cho et al. 2006) family *Xiphophoraceae* (comprising the species *Xiphophora chondrophyla* and *Xiphophora gladiata*, both monoecious hermaphrodites, which used to be classified in the family Fucaceae). Sequences of the internal transcribed spacer region of nuclear ribosomal DNA (ITS) have provided the most complete phylogeny of Fucaceae (Serrão *et al.* 1999). A Pacific origin in the Northern hemisphere has been followed by several switches between oceans, but the history of these is still unclear because some relationships were not clear or concordant between different studies. This is the case for the position of *Ascophyllum nodosum* and *Pelvetia canaliculata*, for example, for which different studies (Serrão et al. 1999, Cho et al. 2006) are not in agreement as to which of them represents the first divergence between the Pacific and Atlantic taxa in the Fucaceae. It is also the case for the relationships between the species of *Fucus*, where phylogenetic data reveal two distinct

clusters (Serrão et al. 1999, Coyer et al. 2006), within which only the species *Fucus serratus* is clearly separable, and are unable to resolve taxa that are clearly different species when compared based on microsatellite allelic frequencies (Billard et al. 2005a; Engel et al. 2005) or a recently derived partially clonal form of *F. vesiculosus* in the Baltic Sea (Tatarenkov et al. 2005), which has been named *F. radicans* (Bergström et al. 2005). The alternate distribution of the character dioecy/hermaphroditism highlights that reproductive system switched several times during speciation in the genus *Fucus*. Of the two distinct clusters within the genus *Fucus*, one, hereafter called lineage 1, comprises the dioecious *F. serratus* distinct from the group of hermaphroditic *F. distichus*, *F. gardneri* and *F. evanescens* and the second one, hereafter called lineage 2, consists of the dioecious *F. vesiculosus* and *F. ceranoides* undistinguishable from the hermaphroditic *F. spiralis* and *F. virsoides*. This lineage 2 includes also asexual or partially asexual entities which may be additional species such as Atlantic *F. cottonii* (Wallace et al. 2004, 2006, Coyer et al. 2006, Engel et al. 2005) and *F. radicans* (Bergström et al. 2005; Tatarenkov et al. 2005). The lack of resolution within each of these *Fucus* clusters has been proposed to be associated to the recent and rapid radiation within the genus (Serrão et al. 1999, Coyer et al. 2006), and may be further complicated by hybridization being possible between taxa with contrasting mating systems, as revealed using microsatellites (Coyer et al. 2002, Engel et al. 2003). Coyer et al. (2006) defend the hypothesis of a north Pacific origin of *Fucus* followed by radiation in the north Atlantic, which would imply hermaphroditism as ancestral state in the genus, from which at least two independent switches to dioecy were derived. However, until now the question of the ancestral reproductive system in *Fucus* is still not clear, even if the evolution of hermaphroditism to dioecy seems the most parsimonious scenario (Billard et al. 2005b).

An additional question that still remains unanswered is why have so many switches between reproductive modes taken place along the evolutionary history of the family Fucaceae? Different ecological conditions are known to favour different mating systems (Takebayashi and Morrell 2001). Fixed abiotic stress might favour selfing of the best adapted genotypes thereby favouring the maintenance of local adaptation as well as reproductive assurance and colonising capacity (Baker 1955, Pannel and Barrett 1998), whereas biotic effects such as the need for competitive ability may favour outcrossing for maintenance of the adaptive capacity towards biotic interactions, maintaining high diversity and avoiding inbreeding depression (reviewed in Uyenoyama et al. 1993). In the Fucaceae, geographical isolation under different environmental conditions, such as when colonising a new ocean system, may have resulted in

contrasting mating systems best adapted to each environment type, or on the other way round, it may have been the mating system itself rather than geographical isolation, that may have provided the opportunity for speciation.

The aim of this study is to investigate the evolutionary history of mating systems in the family Fucaceae, in order to test the hypothesis that evolution proceeded in all cases always from hermaphroditism to dioecy, and to assess whether shifts in mating system might be correlated with major events such as dispersing between different oceans. In order to achieve these goals we will revise existing molecular information and add new datasets from several intergenic spacer regions derived from the *Fucus* chloroplast genome (Pearson unpublished), for the family Fucaceae. Species mating systems will then be mapped on the phylogeny and together with geographic distributional information; these will be used to define evolutionary pathways for the mating systems and the relations between such pathways and important ecological or historical events.

3.3 Material & methods

3.3.1 Taxon sampling and DNA extraction

Nineteen species were sampled among the Fucaceae, and for outgroup its closest relatives which are the southern hemisphere families Hormosiraceae and Xiphophoraceae (Table 1). A northern hemisphere family Himanthaliaceae was initially used to compare its distance levels and potential usefulness but it was excluded afterwards because it was almost unalignable. When possible, samples used were the same individuals as in Serrão et al (1999) or as in Coyer et al (2006) or at least from the same locations. For samples that required new DNA extraction, 20 mg of dried tissue were used in the nucleospin column plant DNA extraction Kit (Macherey-Nagel Düren, Germany) according to the manufacturer's protocol and diluted 1:100.

3.3.2 Chloroplast marker selection

Based on the completely sequenced chloroplast genome of *F. vesiculosus* (Pearson, unpublished) we identified intergenic spacer regions to test for phylogenetic usefulness. Primers were designed in the coding sequences flanking the regions of interest using Primer3

software (Rozen and Skaletsky 2000). The spacer regions that appeared most useful for distinction between species (Table 2) were then selected for sequencing analyses.

Table 1. List of sampled species of the Fucaceae used to sequence the chloroplastic regions thiG-ycf54, psbX-ycf66 and/or rubisco

species	Location	Sample abbrevation	GenBank accession number	Collector, University of Maine Herbarium accession number
Fucus vesiculosus Linnaeus	France, northern Brittany coast, Santec	*F. vesiculosus* France1		C. Daguin & C.R. Engel
	France, northern Brittany, Morlaix river	*F. vesiculosus* France2		C. Daguin
	Northern Portugal coast, Viana do Castelo	*F. vesiculosus* Portugal1		E. Serrão, C. Daguin
	Northern Portugal, Lima river	*F. vesiculosus* Portugal2		C. Daguin
	USA, Maine, Schoodic Point	*F. vesiculosus* USA-Atlantic		E. Serrão
	Canada, Atlantic	*F. vesiculosus* Canada		
Fucus spiralis Linnaeus	France, northern Brittany coast, Santec	*F. spiralis* France1		C. Daguin & C.R. Engel
	France, northern Brittany, Roscoff	*F. spiralis* France2		E. Billard
	Northern Portugal coast, Viana do Castelo	*F. spiralis* Portugal1		E. Serrão, G. Pearson
	Southern Portugal coast, Santa Eulália	*F. spiralis* Portugal2		M. Valente
	USA, Maine, Schoodic Point	*F. spiralis* USA-Atlantic		UMAL280[a]
Fucus ceranoides Linnaeus	France, northern Brittany,Penze river	*F. ceranoides* France1		C. Daguin & E. Billard
	France, southern Brittany, Saint Laurent river	*F. ceranoides* France2		C. Daguin
	UK, Isle of Man, Castletown Harbour 2individuals	*F. ceranoides* UK1 and UK2		UMAL266; J. Jones
	UK, Cornwall, Bude river	*F. ceranoides* and UK3		G. Pearson
	Iceland Kollafjord	*F. ceranoides* Iceland		J. A. Coyer
Fucus virsoides J. Agardh	Italy, Venice,	*F. virsoides* Italy		M. Bastianini
	Slovenia, Adriatic Sea, Cape Debeli	*F. virsoides* Slovenia1		C. Batelli
	Slovenia, Adriatic Sea, San Simon Bay,Isola	*F. virsoides* Slovenia2		C. Batelli
Fucus cottonii	France, northern Brittany, Anse de Terenez	*F. cottonii* France		B. De Reviers
Fucus serratus Linnaeus	Canada, Cape Fourchu, Nova Scotia	*F. serratus* Canada-Atlantic		J.A. Coyer
	Iceland, Sandgerdi	*F. serratus* Iceland		J.A. Coyer
	Norway, Tromso	*F. serratus* Norway		J.A. Coyer
	Sweden Baltic, Oskarhavn,	*F. serratus* Sweden		J.A. Coyer
Fucus evanescens C. Agardh	Norway, Tromso	*F. evanescens* Norway		J.A. Coyer
	Greenland, Nuuk	*F. evanescens* Greenland		J.A. Coyer
	USA, Maine,Appledore Island	*F. evanescens* USA-Atlantic1		J.A. Coyer
	USA, Maine, Schoodic Point	*F. evanescens* USA-Atlantic2y		UMAL276[a]

	Japan, Muroran, Hokkaido	*F. evanescens* Japan		T. Motomura
Fucus gardneri P.C. Silva	USA, Washington, San Juan Island USA,California, Pigeon Point	*F. gardneri USA-Pacific1* *F. gardneri USA-Pacific2*		UMAL259 UMAL265
Fucus distichus Linnaeus	USA, Maine, Chamberlain	*F. distichus USA-Atlantic*		UMAL277[a]
Fucus edentatus	USA, New Hampshire, 2 individuals	*F. edentatus USA-Atlantic1* *F. edentatus USA-Atlantic2*		A. Mathieson
Hesperophycus harveyanus	USA,California,	*H. harveyanus-USA-Pacific*		UMAL251
Pelvetiopsis limitata (Setchell) Gardner	USA, California, Pigeon Point , 2 individuals	*P. limitata USA-Pacific1* *P. limitata USA-Pacific2*		UMAL270
Pelvetia canaliculata (Linnaeus) Decaisne et Thuret	UK, Isle of Man	*Pelvetia canaliculata*		UMAL264
Ascophyllum nodosum (Linnaeus) Le Jolis	France northern Brittany, Roscoff, USA, Maine,Chamberlain	*A. nodosum* France *A. nodosum* USA-Atlantic		UMAL278[a]
Silvetia siliquosa = *Pelvetia siliquosa* (Tseng et C.F. Chang) Serrao, Cho, Boo & Brawley 1999: 215	China, Shandong Peninsula	*S. siliquosa China*		D.L. Duan
Pelvetia fastigiata = *Silvetia. compressa* (J. Agardh) Serrao, Cho, Boo & Brawley 1999:392	USA, California, Pigeon Point	*S. compressa USA-Pacific*		UMAL275[a]
Pelvetia wrightii = *Silvetia. babingtonii* (Harvey) Serrao, Cho, Boo & Brawley 1999: 392	Japan, Muroran, Hokkaido	*S. babingtonii Japan*		UMAL254
Xiphophora chondrophylla (R. Brown ex Turner) Montagne ex Harvey 1855 : 392	Southern Australia, Flinders	*X. chondrophylla Australia*		C. Ashburner
Hormosira banskii	Southern Australia, Sorrento	*H. banski Australiai*		C. Ashburner

The first studied chloroplast region (ThiG-ycf54) was about 250bp long and localised between the genes thiG and ycf54. The second region (psbX-ycf66) was about 280 bp long and located between the genes psbX and ycf66. In addition to the polymorphism tests for phylogenetic purposes, a 550 bp long region including the Rubisco spacer and part of the flanking coding regions RbcL and RbcS was tested for species diagnostic purposes on several individuals of *F. spiralis* and *F. vesiculosus*, using a restriction enzyme (SspI) with a restriction site specific to a sequence found only in *F. spiralis*. In order to check the consistency of this distinction between *F. spiralis* and *F. vesiculosus,* this region was reamplified on 14 individuals of parapatric populations (populations which are not in contact with each other) from each species in Portugal. They were then submitted to restriction by the enzyme SspI, which was expected to cut only for *F. spiralis*.

Table 2: Primers used for PCR amplification as well as for sequencing.

Primer	Direction	Sequence (5'-3')	Approximate annealing position
ThiGycf54F	F	AATTTGCTCAATCAAGCTCACC	ThiG 757
ThiGycf54R	R	CGATAATGTTGTGTTCTTTCACG	ycf54 84
psbX_ycf66F	F	TTGTAGTTTTACCTATTACACTTGC	psbX 52
psbX_ycf66R	R	TGCTCCAAAGACTATATTTATCA	ycf66 2
psaE_ftshF	F	TTCTTAGAATACGTACTTTTGCTCC	psaE(-) 12
psaE_ftshR	R	TTTTTGGGGTTTCATTCTTCA	ftsh 2
ycf39_ilvBF	F	AAAACGCATTAAGGAGTATCA	ycf39(-) 18
ycf39_ilvBR	R	GCTAAATTATTCTAAAGCAAGC	ilvB 39
rbcF2	F	AGTTCTACAATTTGGTGGTG	rbcL 800
rbcR1	R	AGGGTGCGGATCATCTGTC	rbcS 111

3.3.3 DNA amplification and sequencing

Sequencing reactions were carried out directly on polymerase chain reaction (PCR) products. PCRs were performed in 20µL containing 0.1µg/µL bovine serum albumin, 75mM Tris-HCl, 20mM $(NH_4)_2SO_4$, 0.01% Tween®20, 2.5mM $MgCl_2$, 0.25µM of each forward and reverse primer, 200µM of each dNTP, 0.5U Thermoprime Plus *Taq* polymerase (ABgene) and 5µL of diluted DNA. PCRs were run on a PTC200 thermocycler (MJ Research). After an initial denaturation step (95°C, 5min), 'touchdown' PCR was carried out for 5 cycles of 30s at 95°C,

30s at 60°C, reduced by 1°C per cycle for 5 cycles, and 30s at 72°C, followed by 30 cycles of 95°C for 30s, 55°C for 30s and 72°C for 30s and a final 7 min elongation at 72°C. Purified PCR products (Millipore Multiscreen-PCR plates) were sequenced in both directions by using the amplification primers, purified and sequenced on an ABI 3100 capillary sequencer (Perkin-Elmer Applied Biosystems) using the BigDye kit (Perkin-Elmer Applied Biosystems), following the manufacturer's protocol.

3.3.4 Sequence analysis

Sequences were aligned using CLUSTALW (Houssard et al. 1994) as implemented in BIOEDIT 6.0.6 (Hall 1999). Corrections were made by hand because of the numerous insertions/deletions (indels). Polymorphism of sequences within and between clusters was analysed with DnaSP 4.10 (Rozas et al. 2003).

For the phylogenetic reconstruction, intergenic sequences thiG-ycf54 and psbX-ycf66 were concatenated. Indels were coded using the simple coding model (Simmons and Ochoterena 2000) with SeqState (Müller 2005). Aligned sequences were analyzed with Bayesian, maximum likelihood, and parsimony methods. The best evolution model to use in the Bayesian and the Maximum Likelihood analyses was selected using the likelihood ratio test implemented in ModelTest (Posada and Crandall 1998). For ThiG-ycf54 + psbX-ycf66 dataset (referred to as thyGy-psbX), the best model was a Kimura three-parameters with unequal base frequencies K81uf (A=0.4390; C=0.0494; G=0.0724; T=0.4391; A↔C=1.0000; A↔G=1.7321; A↔T=0.2065; C↔G=0.2065; C↔T=1.7321; G↔T=1.0000). For the rubisco spacer dataset, the best model was a general time reversible model with shape parameter of the gamma distribution HKY + Γ (A= 0.36722; C= 0.11114; G= 0.17258; T= 0.34905; A↔C=1.1574; A↔G=1.7057; A↔T=0.1427; C↔G=2.8862; C↔T=3.5705; G↔T=1.0000). These models and substitution rates were used to construct maximum likelihood trees by heuristic searches with random sequence addition and 100 bootstrap values replicates, using PhyML (Guindon and Gascuel 2003). Bayesian analyses were conducted using MRBAYES 3.1 (Huelsenbeck and Ronquist 2003). Each analysis was initiated from a random starting tree and the program was set to run four chains of Markov chain Monte Carlo iterations simultaneously for 1,000,000 generations with trees sampled every 100th generation.

Maximum parsimony (MP) was conducted using PAUP* 4.0 (Beta) using a heuristic search, with tree bisection-reconnection branch swapping, and used 1000 bootstrap replicates

(Swofford 2002). Bayesian estimates of ancestral character states for reproductive system were mapped on the combined thiGy-psbX dataset and on the rubisco spacer dataset using SIMMAP (Bollback 2006).

Table 3. Comparisons between markers for their ability to distinguish species within the genus *Fucus*

		Intra Fucus cluster		*Inter Fucus cluster*
		Lineage 2 *Fves-Fspi/*	*Lineage 1* *Fser/ Feva*	Lineage1/ Lineage2
Number of parsimonious sites /size of the sequence	Nuclear DNA (ITS) mt DNA thiG-ycf54 psbX-ycf66 rubisco	19/821 18/626 1/204 4/281 1/552	14/906 28/626 1/255 1 /281 2/552	40/791 52/626 5/255 6 /281 14/552
Number of segregating sites/ Number of variable sites	Nuclear DNA mt DNA ThiG-ycf54 psbX-ycf66 rubisco	0/19 0/18 (all shared) 0 /1 1 /4 1 /1	2/14 20/28 1 /1 0 0 /2	8/40 19/52 3 /5 4 /6 7 /14
Number of insertions/deletions	Nuclear DNA mt DNA ThiG-ycf54 psbX-ycf66 rubisco	5 0 1 0 0	1 2 0 1 1	7 4 0 1 2

Table 4: Net number of substitutions per site between genera (Da)

	Lineage 2	*Fucus*	*Hesperophycus*	*Pelvetiopsis*	*Pelvetia*	*Ascophyllum*	*Silvetia*
Lineage 1	0.004						
Hesperophycus		0.080					
Pelvetiopsis		0.071	0.049				
Pelvetia		0.095	0.105	0.094			
Ascophyllum		0.093	0.112	0.105	0.093		
Silvetia		0.073	0.095	0.081	0.073	0.051	
Hormosira		0.193	0.206	0.207	0.205	0.214	0.185

3.4 Results

3.4.1 Polymorphism of the cpDNA regions

The three chloroplastic DNA spacers were well conserved within the genus *Fucus* and less polymorphic at the species level than previously studied nuclear (ITS, Serrão et al. 1999) and mitochondrial (Coyer et al. 2006) DNA regions (Table 3). In the thiG-ycf54/psbX-ycf66 dataset (Table 4), the divergence between genera was at least ten times higher than within *Fucus,* ranging from 0.049 net number of substitution per site between *Hesperophycus* and *Pelvetiopsis* to 0.112 between *Ascophyllum* and *Hesperophycus*. This sequence could not be obtained for the *Xiphophora* genus, so for these loci we used only *Hormosira banksii* as outgroup.

The rubisco spacer was however useful for species diagnostic because it presented one mutational difference between the hermaphroditic *F. spiralis* and the two dioecious species *F. vesiculosus* and *F. ceranoides.* Over the 14 *F. spiralis* and 14 *F. vesiculosus* PCR products obtained for this locus, all individuals of *F. spiralis* presented a haplotype cut by the restriction enzyme Ssp1 as expected, whereas none of the *F. vesiculosus* was. Given the problematic distinction between the species Fv and Fspir based on morphology and the occurrence of intermediate morphologies and intermediate genotypes, we chose to screen the rubisco spacer for individuals whose species name we can identify based on microsatellite genotypes. One individual in genbank (AY246553) was not consistent with this difference but since it does not come from a publication and it is not reported where this individual was collected and identified, we choose to include only those that we could certify the genetic entity for.

3.4.2 Phylogenetic analyses

All methods of phylogenetic analyses gave the same tree topologies, thus only one tree is shown for the concatenated thiGy-psbX dataset (Fig. 1) and one for the rubisco spacer (Fig. 2) for which sometimes different individuals had been sequenced. The cpDNA intergenic markers confirmed the existence of the two clusters of *Fucus* already mentioned by Serrão et al (1999) and Coyer et al (2006) in their analyses based on nuclear and mitochondrial markers respectively. These clusters correspond to lineage 1 of Serrão et al (1999) comprising the dioecious *F. serratus* and the hermaphroditic *F. evanescens*, *F. distichus*, *F. edentatus* and *F.*

gardnerii, and to lineage 2 comprising the dioecious *F. vesiculosus* and *F. ceranoides* and the hermaphroditic *F. spiralis* and *F. virsoides*. On the other hand the Bayesian analysis of the Rubisco shows the genus *Fucus* as not monophyletic, with the *Pelvetiopsis/Hesperophycus* cluster grouping with the *F. serratus.* However, this was very poorly supported with the ML analysis (50.8%) and was unresolved with the MP analysis. The hermaphroditic genus *Silvetia*, as described in Serrao et al (1999) and the dioecious species *Ascophyllum nodosum* are now clustering together revealing a common ancestor and a more recent divergence than what was suggested based on the ITS data (Serrão et al 1999). This result is well-to-maximally supported depending on the Maximum parsimony (MP bootstrap = 0.71), maximum likelihood (ML bootstrap = 0.92) or Bayesian analysis (posterior probability = 1.0). Moreover, this cluster is always very-well-to-maximally supported when the analysis is performed with the rubisco marker (Fig 2). The *Pelvetia* genus, from the Atlantic, branched with the cluster containing the genera *Hesperophycus*, *Pelvetiopsis* and *Fucus,* confirming the nuclear ITS results (Serrão et al. 1999) that show it as the first divergence within this group, a pattern that was not supported with the chloroplast gene psaA (Cho et al 2006).

3.4.3 Ancestrality of sexual phenotypes

The phylogeny obtained provided a framework to test for the evolution of sexual characters within the Fucaceae and more precisely to test for the ancestral character for reproductive mode in the genus *Fucus*. The Bayesian analysis performed with SIMMAP on the concatenated sequences thiG-ycf54/psbX-ycf66 revealed a probability of 0.86 that hermaphroditism could be the ancestral type in the genus *Fucus*.

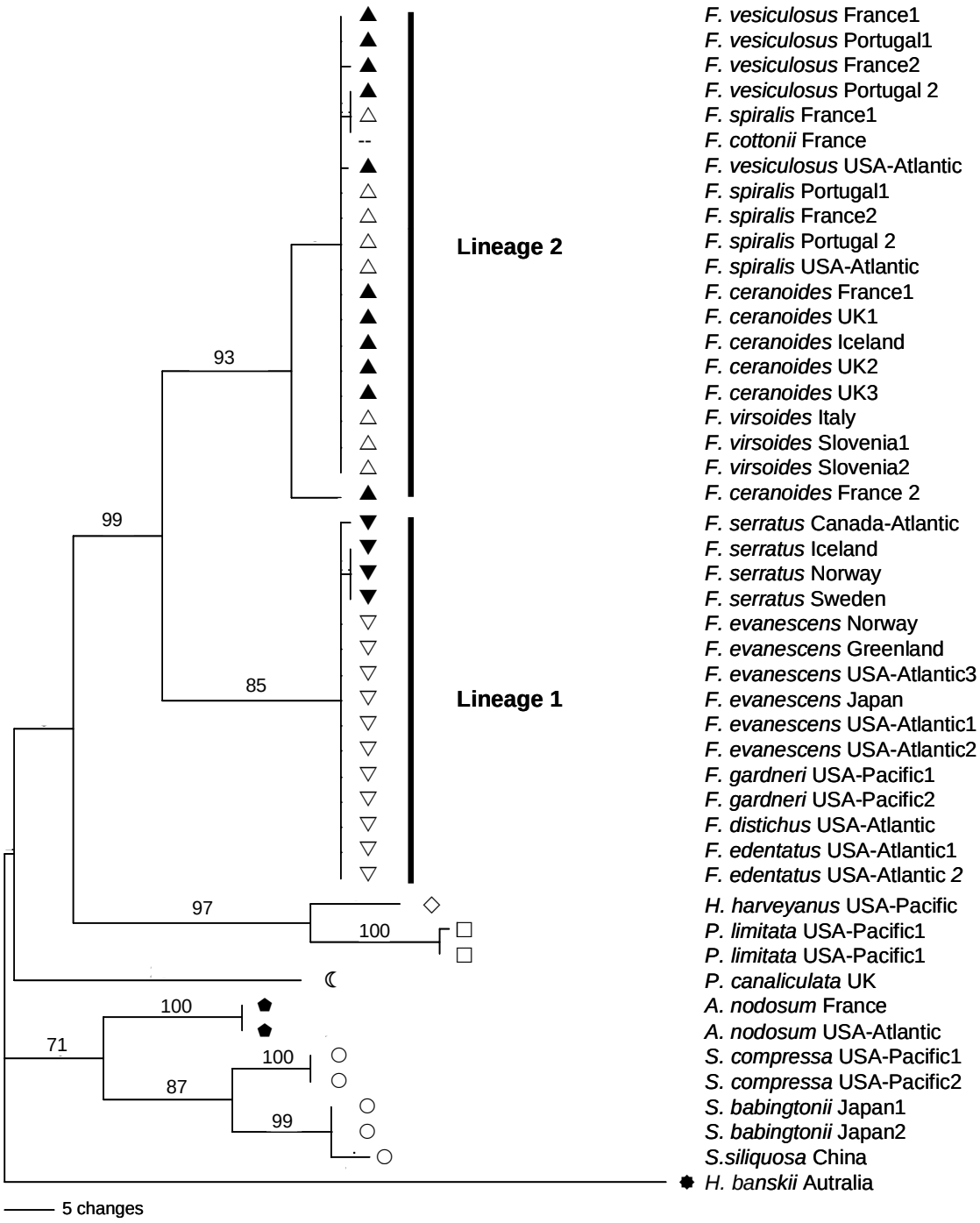

Figure 1, Maximum Parsimony analysis inferred from chloroplast intergenic regions thiG-ycf54 and psbX-ycf66 concatenated Each symbol represents a different genus except for the two lineages of the *Fucus* genus which have the same symbol but inverted. Filled symbols represent dioecious taxa and open ones represent hermaphroditic taxa. F. cottonii is represented with dots, as this species is mainly reported as asexual. Numbers are bootstrap values, only values greater than 70 are indicated

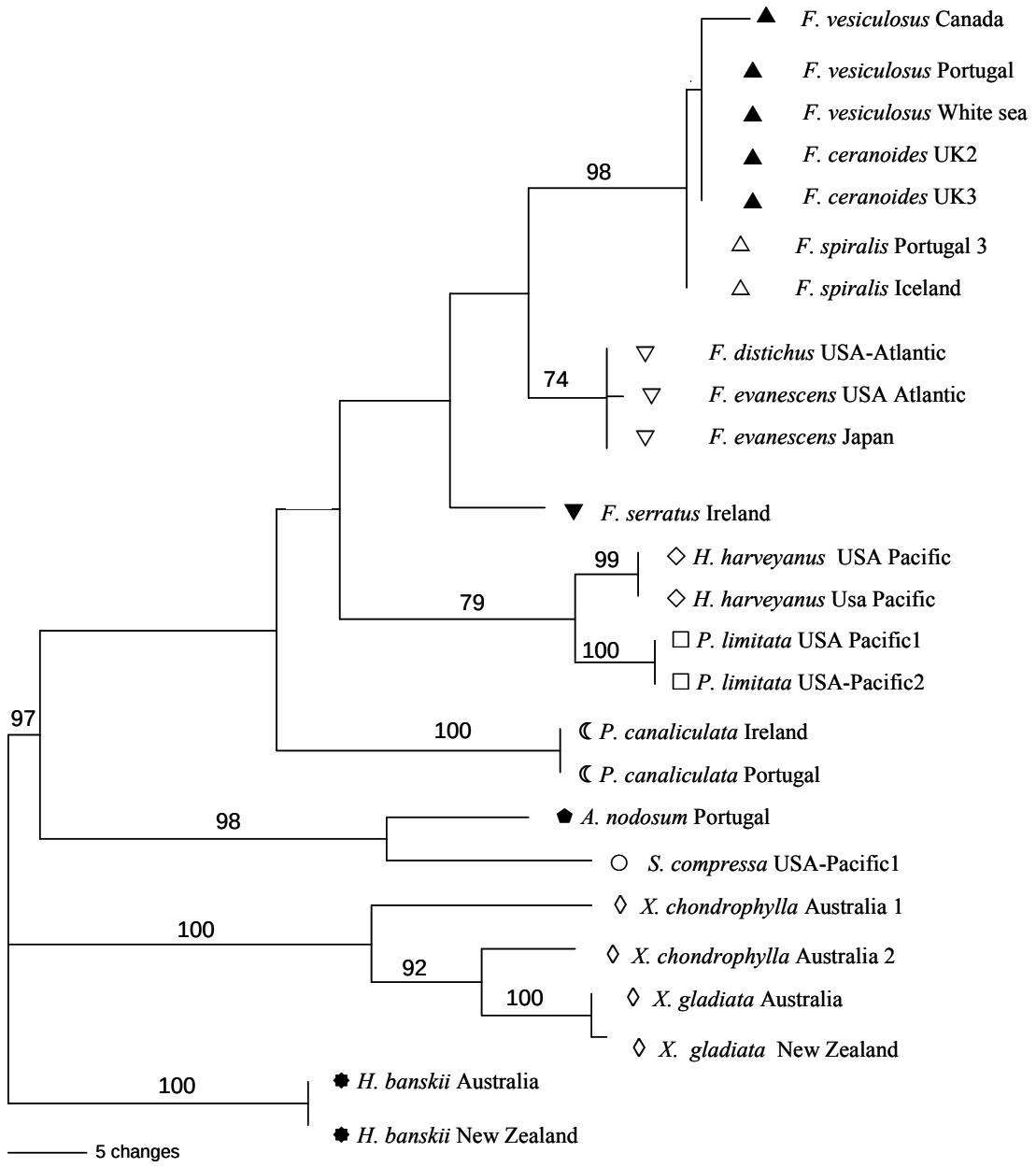

Figure 2: Maximum Parsimony analysis inferred from the Rubisco spacer and parts of the Rubisco Large sub-unit and small sub-units. The same symbols as in Figure 1 are used to figure the mating. Numbers are bootstrap values, only values greater than 70 are indicated.

3.5 Discussion

The chloroplastic markers showed a lower degree of polymorphism than the mt-spacer previously used by Coyer et al (2006) making them perhaps more useful at the inter-generic than at the intra-generic level, as seen by the low resolution within the *Fucus* lineages.

However, it is noteworthy that the rubisco spacer presented one mutational difference between the hermaphroditic *F. spiralis* and the two dioecious species *F. vesiculosus* and *F. ceranoides* thus providing a molecular discrimination between these species, although the hybridization occurring between these species (Burrows and Lodge 1953; Engel et al. 2005; Gard 1910) may induce some atypical variation when they are in contact.

This study based on chloropastic markers over 6 genera of Fucaceae is in part in accordance with the previous study of the family Fucaceae based on nuclear ITS (Serrão et al. 1999). Within the genus *Fucus*, the existence of two main lineages is also supported, as it was with the mitochondrial and nuclear markers (Coyer et al. 2006; Serrão et al. 1999). The existence of two genera in what was previously called the genus Pelvetia, now *Silvetia* in the Pacific and *Pelvetia* in the Atlantic, appears also well supported and is confirmed by their level of divergence of the same order of magnitude as the other genera. The main difference from the previous results on the phylogeny of the Fucaceae consists in the resolution of the most basal node, between *Silvetia*, *Ascophyllum* and the rest of Fucaceae. This was a trichotomy with the full ITS dataset available (Serrão et al. 1999), and restricting the analyses to one individual per taxa this suggested, although with low support, that *Ascophyllum* was an initial divergent lineage separated from a common ancestor to *Silvetia* and all remaining Fucaceae (Serrão et al. 1999). Our new dataset now provide good statistical support to falsify this inference, and concluding that *Ascophyllum* and *Silvetia* are a monophyletic clade, which is also supported by the chloroplast psaA gene tree in a recent phylogeny of the order Fucales (Cho et al 2006).

The new inference that the dioecious genus *Ascophyllum* is derived from a common ancestor with the hermaphroditic *Silvetia*, rather than being the single most basal divergence in the family, now provides more support to the hypothesis of hermaphroditism as the ancestral character state in the family. Interestingly, the clustering of the dioecious *A. nodosum* with the hermaphroditic genus *Silvetia* now suggests that dioecy may have appeared independently in the different branches of Fucaceae, but it does not anymore imply the need for a reversal of dioecy to hermaphroditism, as was suggested by Serrão et al (1999). Furthermore, the hermaphroditic *Pelvetia* is the only sister group of the cluster composed by the hermaphroditic genera *Hesperophycus*/*Pelvetiopsis* and the genus *Fucus* , which also supports more confidently the hypothesis of hermaphroditism being the ancestral state in the genus *Fucus*. These results are congruent with the hypothesis for the *Fucus* genus (Billard et

al. 2005b) of an evolution from hermaphroditism to dioecy with few major genes involved, via gynodioecy (Charlesworth 1999; 2002).

The Pacific origin of the Fucaceae first suggested by Clayton (1984) is well supported by the existence of the Australasian *Hormosira* and *Xiphophora* genera as sister groups and is commonly accepted (Cho et al. 2006; Coyer et al. 2006; Serrão et al. 1999). The new resolution of *Ascophyllum* implies also that the two most primitive Fucaceae of the Atlantic Ocean, *Ascophyllum* and *Pelvetia*, may have derived from independent crossings from the Pacific to the Atlantic, and thus suggest that, at least three switches between oceans have occurred; once by the ancestor to *Ascophyllum*, which may have been accompanied by the appearance of dioecy, once by the ancestor to *Pelvetia* and al least once in the genus *Fucus*. Concerning the genus *Fucus*, Coyer et al (2006) have suggested two possible scenarios. The first one implies one ancestor for each lineage occurring in the Pacific Ocean and deriving into the current hermaphroditic and dioecious species while crossing from Pacific to Atlantic Ocean. The second scenario postulates only one common ancestor in the Pacific radiating into the two lineages during its dispersion through North Atlantic and then deriving into the dioecious and hermaphroditic species in the two lineages. In this case, *F. spiralis* from the Pacific coast is suggested to be a recent human introduction from Atlantic (Coyer et al. 2006; Lüning 1990; Serrão et al. 1999), and indeed it was only first reported in the Pacific three decades ago ((Norris and Conway 1974)), although it had been suspected to be present there previously (see references in Norris and Conway 1974).

The current distribution pattern of different reproductive systems among Fucaceae may not be easily explained by major historical/ecological events, except the common pattern that in this family, dioecy occurs only in the Atlantic. It is however unknown whether dioecy appeared only in the Atlantic or might have dioecious species become extinct in the Pacific Ocean? In either case the result is that all dioecious species of the family are now Atlantic endemics, which is suggestive of evolutionary advantages of this reproductive mode in the Atlantic versus the Pacific. One possible hypothesis suggested by this pattern might be the high complexity of the coastlines along many Atlantic regions creating extensive sheltered intertidal zones – these conditions favour high success of external fertilization (reviewed by Pearson and Serrão 2006), a condition that is essential for long-term survival of externally fertilizing dioecious species, thus overcoming the need for the reproductive assurance (Baker 1955) that is provided by self-compatible hermaphroditism. According to this hypothesis

hermaphroditic species would have acted as pioneers in colonizing new habitats in the Atlantic but then might have been or are being over-competed and restricted to local distribution limits (e.g., along the vertical distribution) by the evolution of dioecious relatives in habitats that provide adequate conditions for successful dioecious reproduction (i.e., sheltered and dense populations). Indeed, selfing is expected to reduce the effective population size and consequently the level polymorphism and the efficacy of selection. Several studies using different markers converge to show that a selfing mating system considerably reduces species-wide diversity (Hamrick and Godt 1996, Nybom 2004, Glémin et al. 2006). This seems to be the case in the *Fucus* species found in the Atlantic, with the hermaphroditic species *F. spiralis* being a predominantly selfing species and showing lower diversity than the dioecious related species *F. vesiculosus* and *F. serratus* (Engel et al. 2005; Billard et al 2005b; Perrin et al. 2007).

Nevertheless, hermaphroditism in the Fucaceae is maintained in many taxa and may represent a good means of dispersal by providing reproductive assurance when one or a few individuals reach a new region. It may also be important for persistence in physically stressful habitats in that the ability to self contributes to maintaining locally adapted gene complexes, and data available to date indeed suggest that selfing may be predominant in at least some hermaphroditic Fucaceae (e.g., Billard et al. 2005, Engel et al. 2005, Perrin et al. 2007, but see also Coleman & Brawley 2005). Indeed, the most physically stressed habitats, like upper shores, are usually colonized by hermaphroditic species such as *F. spiralis, F. distichus*, or *Pelvetia canaliculata*. Moreover, cases of asexual reproduction in *Fucus*, mostly occur in non suitable habitats like the newly described *F. radicans* in the brackish Baltic Sea (Tatarenkov *et al.*, 2005), where the low salinity may limit the fertilization success by reducing the motility and viability of gametes (Serrão et al. 1996) and many salt-marsh variants in this family (Mathieson et al. 2006). In our scenario we consider that the hermaphroditic species will tend to be preferentially self-fertilised and to occur in areas that are geographically or ecologically marginal compared to related dioecious outcrossing species. The question then arises of why is external fertilisation maintained in these selfing hermaphrodic species? Future experimental studies could be planned to address this question.

In conclusion, this study revises the evolutionary history of the Fucaceae and strongly supports the hypothesis of dioecy evolving from hermaphroditism in the Fucaceae, and that contrarily to previous hypotheses; dioecy might never have reversed towards hermaphroditism.

3.6 Acknowledgments

We thank all the collectors, particularly Jim Coyer who provided many samples, and Susan Brawley for the use of the sample collection from Serrão et al. (1999), and Regina Cunha for help in the analyses. This study was funded by the NoE Marine Genomics Europe (GOCE-CT-2004-505403, WP6, algal node) and the Portuguese Science Foundation (FCT with support from FEDER and ESF).

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This new phylogenetic study, based on the analysis (maximum parsimony, maximum likelihood and Bayesian) of chloroplastic markers allowed us to suggest that, like in higher plants, dioecy must have evolved from hermaphroditism. Contrarily to previous studies, no sign indicates a possible reversion from dioecy to hermaphroditism was found.

The markers used appeared to be useful tool at the generic level, resolving for example the uncertainties remaining in the phylogeny of Serrão et al. (1999) concerning the relationships between the genera *Ascophyllum* and *Silvetia*. Moreover, the Rubisco marker enabled for the first time the distinction between the species *F. vesiculosus* and *F. spiralis*.

However, the relationships between species of the genus *Fucus* remains poorly resolved, particularly within the lineage 2 composed, among others, of *F. spiralis*, *F. vesiculosus* and *F. ceranoides*. This last species is restricted to estuaries and although generally considered as dioecious, some hermaphroditic individuals have been reported in the literature (Hamel, 1939). Knowing the hybridization possibilities between *F. spiralis* and *F. vesiculosus* (Engel et al., 2005) and the phenotypic polymorphism occurring in *F. vesiculosus* (*F. vesiculosus var evesiculosus* for example) we aimed to check the existence of three distinct genetic entities within this group.

This study will be the subject of the second chapter (Article 2) and was published in the *Journal of Phycology* (2005)

ARTICLE 2

Genetic isolation between three closely related taxa: *Fucus vesiculosus*, *F. spiralis* and *F. ceranoides*

4 GENETIC ISOLATION BETWEEN THREE CLOSELY RELATED TAXA: *FUCUS VESICULOSUS, F. SPIRALIS* AND *F. CERANOIDES*

Billard, E[1,2]., Daguin, C[1,2]., Pearson, G.[2], Serrão, E.[2], Engel, C.[1] and Valero, M.[1] (2005) Genetic isolation between three closely related taxa: *Fucus vesiculosus, F.spiralis,* and *F. ceranoides* (Phaophyceae). *Journal of Phycology*, 41, 900-905

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4.1 Absract

All traditional markers, both phenotypic and phylogenetic, have failed to discriminate between the taxa composing the *F. vesiculosus* L. / *F. spiralis* L. / *F. ceranoides* L. species complex particularly in Brittany (France), so we used five microsatellite markers to compare the allelic frequencies of populations of the three taxa in this region. The aim of this study was to assess whether the different populations were grouped according to their geographical location, their habitat (open coast *vs.* estuary) or their *a priori* taxonomic assignment. Species-specific alleles were identified at one locus, demonstrating the utility of microsatellite markers for recognizing the three taxa in Brittany. Moreover, our results clearly supported the separation of *F. vesiculosus*, *F. spiralis* and *F. ceranoides* into distinct species, independently of geography. We also identified genetic differentiation between estuarine and coastal populations of *F. vesiculosus*.

Keywords: genetic differentiation, fucoid, genetic taxonomy, microsatellite, Phaeophyceae, species complex

Abbreviations: UPGMA = unweighted pair group method using an arithmetic average, NJ = neighbour joining, CA = correspondence analysis

4.2 Introduction

Within the genus *Fucus*, the three taxa *F. vesiculosus* L., *F. spiralis* L. and *F. ceranoides* L. are closely related, possibly as the result of a recent radiation (Leclerc et al. 1998, Serrão et al. 1999). Although commonly regarded as separate species (e.g., ALGAEBASE, http://www.algaebase.org), to date neither phenotypic (Burrows and Lodge 1951; Pérez-Ruzafa et al. 1993) nor genetic (Serrão et al. 1999) characteristics have been able to differentiate between *F. vesiculosus*, *F. spiralis* and *F. ceranoides*. Indeed, the morphological characters of these species present no clear discontinuities (Pérez-Ruzafa et al. 1993). On the other hand, on the basis of their study of chemical phenotypes using pyrolysis mass spectrometry, Hardy et al. (1998) considered that *F. vesiculosus*, *F. serratus* L., *F. spiralis* and *F. ceranoides* are distinct species. However, given that these species can be found in different habitats, morphological and chemical phenotypes may depend on environmental conditions, and without transplants across habitats (lower *versus* upper shore, or rocky shores *versus* soft sediment estuarine zones), observed phenotypic differences may not reflect phylogenetic relationships. *Fucus vesiculosus* in particular displays high phenotypic plasticity, often correlated with biological and physical aspects of the habitat (e.g. Knight and Parke 1950, Niell et al. 1980, Kalvas and Kautsky 1993; Pérez-Ruzafa et al. 1993).

In Brittany, variability of molecular markers such as ITS sequences has been reported to be extremely low for *F. vesiculosus*, *F. spiralis* and *F. ceranoides* (Leclerc et al. 1998). Relationships within the clade containing these three species collected on both sides of the North Atlantic were not resolved (Serrão et al. 1999). Moreover, despite the lack of ITS resolution, the sequences of Brittany samples of these three *Fucus* species all clustered together. Although bootstrap support was low (63%), this result suggested that geography might be a more important predictor of relatedness than species differences, further questioning their distinctness as species.

The aim of this study was to use highly polymorphic microsatellite markers (developed by Engel et al. 2003) to assess the genetic distinctness of *F. vesiculosus*, *F. spiralis* and *F. ceranoides* within the Brittany region by comparing allele frequencies and thereby possibly identifying species-specific genetic markers. Although not traditionally utilized for taxonomic purposes, these markers have been shown to be useful for distinguishing closely related species (Muir et al. 2000). Indeed different microsatellite

markers were used very recently to distinguish estuarine forms of *F. vesiculosus* and *F. spiralis* (Wallace et al. 2004).

4.3 Materials and Methods

Table 1: Sampled populations of the three *Fucus* taxa. At each location, the distance between replicate samples was between 15 m to 150 m. *F. vesiculosus* and *F. spiralis* samples from Santec and Brignogan were collected attached to the rocks in open coastal habitats while all other samples were collected in muddy estuaries.

Species	Code	Location	Geo-position	Sample size
F. spiralis	FsSant1	Santec (NB)	48°42N, 4°03'E	22
	FsSant2	Santec (NB)	48°42N, 4°03'E	22
	FsSant3	Santec (NB)	48°42N, 4°03'E	22
	FsSant4	Santec (NB)	48°42N, 4°03'E	22
	FsBrig1	Brignogan (NB)	48°40'N,4°18'E	24
	FsBrig2	Brignogan (NB)	48°40'N,4°18'E	24
F. vesiculosus	FvSant1	Santec (NB)	48°42N, 4°03'E	20
	FvSant2	Santec (NB)	48°42N, 4°03'E	21
	FvSant3	Santec (NB)	48°42N, 4°03'E	22
	FvSant4	Santec (NB)	48°42N, 4°03'E	22
	FvBrig1	Brignogan (NB)	48°40'N,4°18'E	24
	FvBrig2	Brignogan (NB)	48°40'N,4°18'E	24
	FvMorl1	Morlaix river (NB)	48°37'N, 3°51'E	24
	FvMorl2	Morlaix river (NB)	48°37'N, 3°51'E	24
	FvPenz1	Penzé (NB)	48°38'N, 3°57'E	24
	FvPenz2	Penzé (NB)	48°39'N, 3°57'E	24
F. ceranoides	FcPenz1	Penzé (NB)	48°38'N, 3°57'E	23
	FcTeren	Terenez (NB)	48°40'N, 3°51'E	20
	FcSLau1	Saint Laurent (SB)	47°55'N, 3°70'E	24
	FcSLau2	Saint Laurent (SB)	47°55'N, 3°70'E	24

NB: North Brittany, SB: South Brittany

4.3.1 Sampling

Twenty populations were sampled in the Brittany region, France (Table 1), in order to investigate whether genetic distances were greater between taxa, between habitats (estuary *versus* open coast populations) or between locations (geographic distance). Taxon determination was primarily based on the following morphological characters: wide thalli and presence of vesicles for *F. vesiculosus*; wide thalli, receptacles with a rim of sterile tissue and absence of vesicles for *F. spiralis*; and very thin thalli with acute and branched receptacles and absence of vesicles for *F. ceranoides*. The sampling of the three taxa was also based on their habitat characteristics: *F. spiralis* inhabits mainly open coastal rocky shores and *F. ceranoides* occupies muddy estuaries, whereas *F. vesiculosus* can be found in both habitats.

F. vesiculosus was therefore sampled from both open coast and estuarine locations in order to test for genetic differentiation among habitats (Table 1). Approximately 100 individuals per taxon (and habitat type in *F. vesiculosus*) were collected for genotyping (Table 1). For each taxon, the sampling design included at least two different locations separated by tens to hundreds of kilometres and several replicates for most of the locations (Table 1). Individuals from the different taxa co-occurred at three of the chosen locations: *F spiralis* grew with *F. vesiculosus* at Santec and at Brignogan; and *F. ceranoides* was found with *F. vesiculosus* at Penzé (Table 1). In open coastal populations, *F. spiralis* and *F. vesiculosus* individuals were found attached to rocks, whereas estuarine populations of *F. ceranoides* and *F. vesiculosus* were found either in mud or attached to rocks. Vegetative tips (2 – 3 for each individual) were stored in silica gel for future molecular analyses.

4.3.2 DNA extraction, PCR reaction and genotyping

DNA for genotyping was extracted from ca. 4 mg of dried tissue using the Nucleospin® Multi-96 plant kit (Macherey-Nagel) according to the manufacturer's protocol and diluted 1:500. Polymerase chain reactions (PCR) and electrophoresis of PCR products for loci L20, L38, L94, L58, L78 were performed as described in Engel et al. (2003).

4.3.3 Data analysis

For each population, allele frequencies were calculated at all five loci and a correspondence analysis (CA) based on these data was performed using the AFC procedure implemented in the GENETIX software (Belkhir 2003). All individuals with missing data at one or more loci were excluded; CA analysis was thus performed on 358 of the 456 sampled individuals. Nei's genetic distances (Nei 1972) were computed for each pair of populations and distance trees were obtained using two different reconstruction methods, neighbor joining (NJ) and UPGMA (unweighted pair group method using an arithmetic average) using PHYLIP software (Felsenstein 1986). Robustness of the topology was tested using 1000 bootstrap re-samplings. Since comparisons are being made between species, we used Nei's distance because it is more appropriate for long-term evolution when populations diverge due to drift and mutation (Weir 1996, p. 197). Moreover, Nei's genetic distance is the most commonly used genetic distance and therefore has been chosen to allow comparison with other work (see review of Sites and Marshall, 2003). From the matrix of Nei's distances, average distances within and between taxa were then computed using MEGA version 2.1 (Kumar et al. 2001).

4.4 Results and discussion

All five loci developed for the two taxa *F. vesiculosus* and *F. spiralis* (Engel et al. 2003) were easily cross-amplified in *F. ceranoides* as expected from the close phylogenetic relationships among these three taxa. Contrary to the study of Wallace et al. (2004), in which all four microsatellite loci developed were polymorphic in both *F. spiralis* and *F. vesiculosus*, the five loci used here showed contrasting levels of polymorphism depending on the taxon. All five loci were polymorphic in *F. vesiculosus*, but most loci were fixed for one allele (or nearly fixed with the allele frequency of the most common allele greater than 0.95) for the other two taxa (Table 2). Nevertheless, due to the fixation for different alleles, *F. spiralis* and *F. ceranoides* samples could be easily distinguished with the monomorphic loci. The fixed (or nearly fixed) alleles for *F. spiralis* and *F. ceranoides* were, respectively, alleles 169 and 190 at locus L38; alleles 145 and154 at locus L94 and alleles 122 and 131 at locus L78. Moreover, at locus L78 alleles 122 (typical of *F. spiralis*) and 131 (typical of *F. ceranoides*) were very rare (observed at a frequency less than 0.05) within *F. vesiculosus*, making this locus diagnostic for identifying the three different taxa in Brittany (Table 2). Although ITS sequences failed resolve these taxa, and even showed geographic clustering (Serrão et al. 1999), we clearly show here that microsatellite loci can be used to distinguish the three taxa in the '*F. vesiculosus/ F. spiralis/ F. ceranoides'* clade within Brittany.

Three groups of individuals were identified by CA: the first axis enabled us to differentiate *F. spiralis* from the two other taxa, while the second axis separated *F. ceranoides* from *F. vesiculosus* (Fig. 1). Estuarine individuals of *F. ceranoides* and *F. vesiculosus* did not group together; rather, *F. ceranoides* individuals from Northern and Southern Brittany grouped together to the exclusion of other estuarine individuals from Northern Brittany. Therefore, this analysis shows a clear separation along taxonomic lines. The distribution along the first axis (Fig. 1) was more scattered for individuals identified as *F. vesiculosus* than for the two other species with some intermediate points coming from FvSant2 and FvBrig2 populations (Table 1)

Table 2: Allele frequencies at five microsatellite loci. Frequencies were computed for the total sample within each of the three species: *F. spiralis* (*Fsp*), *F. vesiculosus* (*Fve*) and *F. ceranoides* (*Fce*)

Locus 20			
	Fsp	*Fve*	*Fce*
N	131	226	84
allele size			
120	_	0.013	_
129	_	0.002	_
135	_	0.004	_
138	_	0.124	0.006
141	0.004	0.126	_
144	_	0.175	_
147	0.920	0.086	0.738
150	_	0.049	0.060
153	0.076	0.208	0.179
156	_	0.144	0.018
159	_	0.031	_
162	_	0.022	_
165	_	0.002	_
168	_	0.002	_
171	_	0.007	_
174	_	0.002	_
180	_	0.002	_

Locus 58			
	Fsp	*Fve*	*Fce*
N	136	220	79
allele size			
105	_	0.014	0.000
107	1.000	0.357	0.006
109	_	0.296	0.013
111	_	0.314	0.032
113	_	0.014	0.006
115	_	0.002	0.817
117	_	0.002	0.127
119	_	0.002	0.000

Locus 94			
	Fsp	*Fve*	*Fce*
N	136	219	75
allele size			
127	_	0.002	_
136	_	0.005	_
145	0.996	0.018	_
148	_	0.034	_
154	0.004	0.434	1.000
157	_	0.007	_
160	_	0.279	_
163	_	0.027	_
166	_	0.135	_
169	_	0.048	_
175	_	0.011	_

Locus 38			
	Fsp	*Fve*	*Fce*
N	136	211	89
allele size			
139	_	0.002	_
163	_	0.019	_
166	_	0.010	_
169	1.000	0.251	_
172	_	0.043	_
175	_	0.005	_
181	_	0.010	_
190	_	0.495	0.994
193	_	0.031	_
196	_	0.026	0.006
199	_	0.088	_
205	_	0.002	_
208	_	0.012	_
211	_	0.005	_
226	_	0.002	_

Locus 78			
	Fsp	*Fve*	*Fce*
N	136	195	83
allele size			
113	_	0.005	_
119	_	0.005	_
122	0.956	0.010	_
128	_	0.005	_
131	_	0.013	1.000
132	0.011	_	_
134	_	0.005	_
137	0.022	0.021	_
140	_	0.005	_
143	_	0.056	_
146	_	0.003	_
149	_	0.123	_
152	_	0.226	_
155	0.011	0.190	_
158	_	0.051	_
161	_	0.241	_
164	_	0.028	_
167	_	0.010	_
176	_	0.003	_

N : sample size

This last result could be explained by the higher level of polymorphism within *F. vesiculosus* and also by the existence of genetically intermediate individuals resulting from gene flow between *F. vesiculosus* and the other taxa. Specifically, one *F. vesiculosus* individual collected in the Penzé river tended to cluster with *F. spiralis* rather than with *F. vesiculosus* (Fig. 1: open triangle in the middle of dark squares). The genotype of this individual identified as *F. vesiculosus* in the field matched with this taxon at the diagnostic locus 78 while for all four other loci, its genotype was typical of *F. spiralis*. This individual may be the result of hybridization between *F. vesiculosus* and *F. spiralis*.

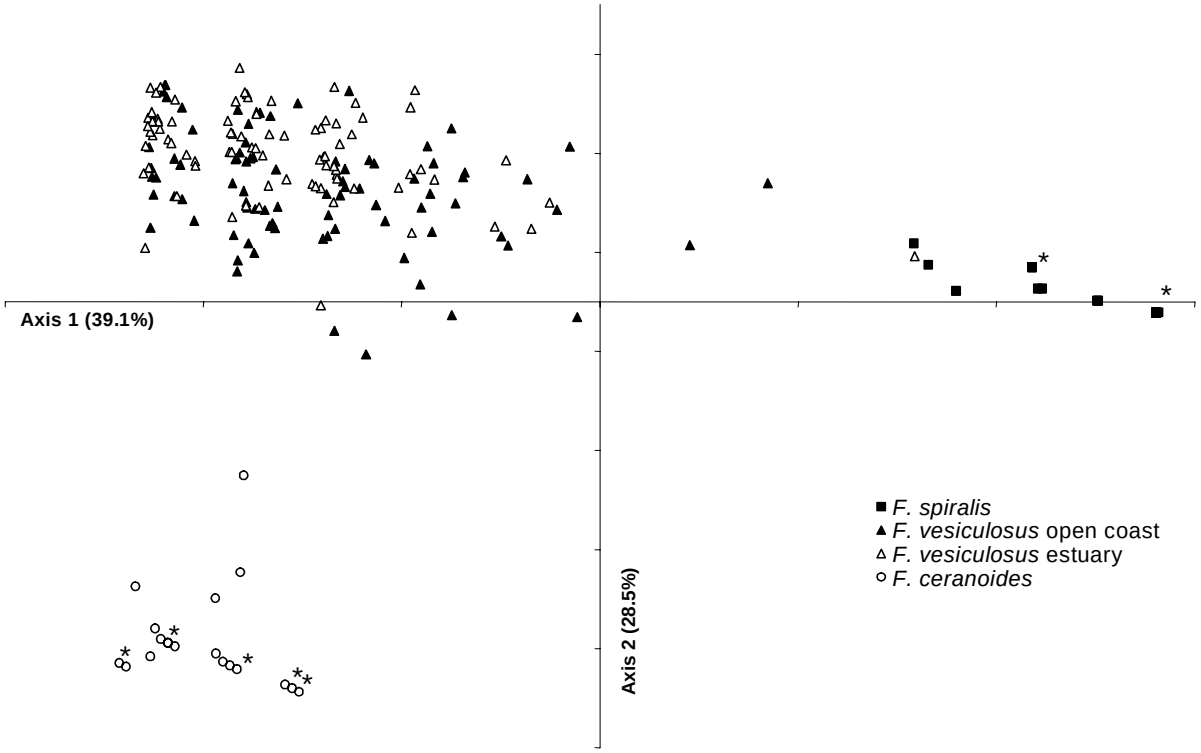

FIG. 1: Correspondence Analysis based on allele frequencies of *Fucus spiralis*, *F. vesiculosus* and *F. ceranoides* populations in Brittany calculated at five microsatellite loci. Plot of individuals. Inertia of each axis is given in parentheses. * more than one individual is superimposed.

UPGMA and NJ phylogenetic reconstruction methods (Fig. 2A and 2B respectively) did not produce exactly the same tree topology. Nevertheless, both showed that, in general, F. vesiculosus and F. ceranoides were genetically closer to each other than either was to *F. spiralis* (Fig. 2). In the UPGMA tree, three distinct clades were well supported by bootstrap

values greater than 90%, confirming that individuals were grouped according to their taxonomic assignment and not by their geographic origin (Fig. 2A). Indeed, *F. ceranoides* individuals from Penzé did not cluster with *F. vesiculosus* individuals from the same area, but with individuals of the same taxon collected more than 100 km away (*e.g.* Saint-Laurent). We also found that *F. vesiculosus* individuals were split into two different clades corresponding to their habitat (the estuarine clade being supported by a bootstrap value of 78%), revealing differentiation between estuarine and open coast populations (Fig. 2A).

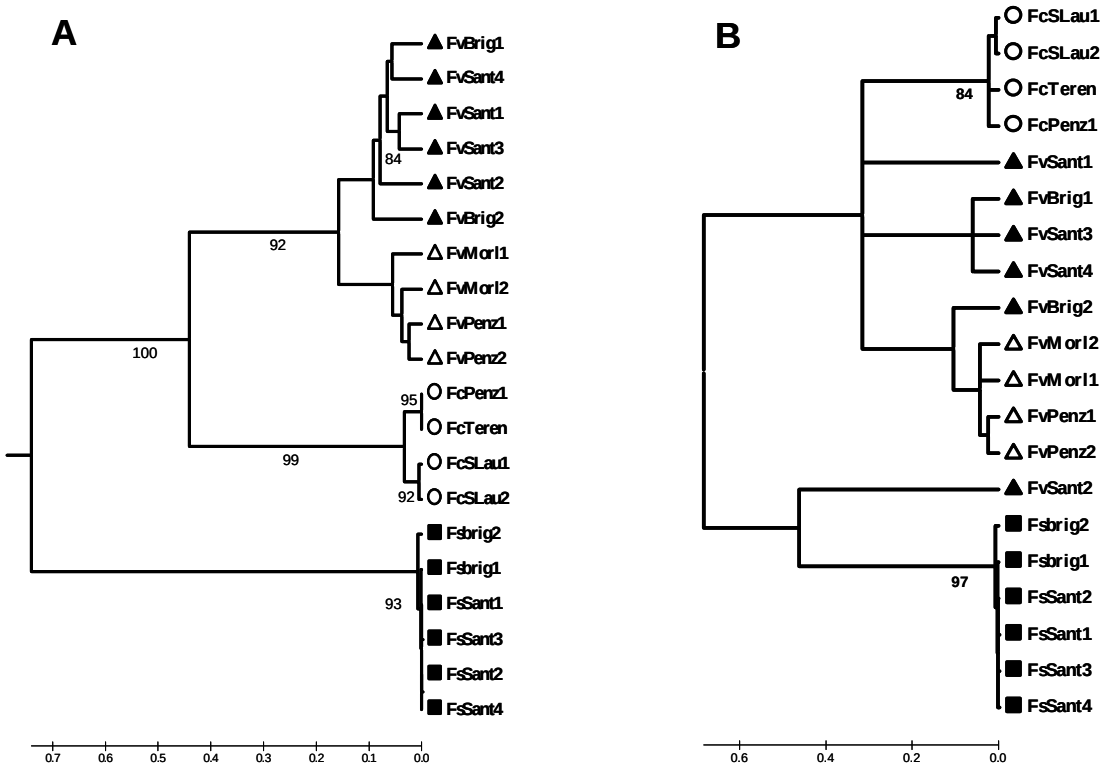

FIG. 2: UPGMA (A) and Neighbor Joining (B) distance tree of populations of *Fucus spiralis*, *F. vesiculosus* and *F. ceranoides*, based on Nei's distances (see scale bar). Only bootstrap values superior or equal to 80% from 1000 replicates are shown. See Fig. 1 legend for symbols.

In the NJ tree, two well defined clades (bootstrap values greater than 88%) were again found for *F. ceranoides* and *F. spiralis* (Fig. 2B). However, contrary to UPGMA, the latter reconstruction method was not able to resolve the relationships within *F. vesiculosus*, in particular among open coast populations. The scattering of *F. vesiculosus* populations through the tree revealed that this taxon is not as genetically cohesive as the other two taxa. Like the

CA analysis, this result suggests that reproductive isolation may not be complete within *F. vesiculosus* with the occurrence of intermediate individuals. The occurrence of individuals genetically intermediate between *F. spiralis* and *F. vesiculosus* has been recently demonstrated in the North West Atlantic (Maine, USA, Wallace et al. 2004) and in two distant regions of the North East Atlantic coast (Cape Gris-Nez in northern France and Viana do Castelo in northern Portugal, Engel et al. in press). Consequently, interspecific gene flow occurring after divergence of the two taxa may be responsible for this pattern, particularly in regions where both species co-occur. If so, the pattern of mosaic hybridization between these two species appears to be a very different process to that observed between *F. serratus* and *F. evanescens* by Coyer et al. (2002) for which hybridization was restricted to a zone of recent contact.

Average Nei's genetic distances confirmed that the three morphological entities correspond to different genetic entities. Average distances between taxa (1.94 between *F. spiralis* and *F. ceranoides*; 1.30 between *F. spiralis* and *F. vesiculosus*, and 0.88 between *F. vesiculosus* and *F. ceranoides*) were ca. three to two hundred times higher than within taxa (0.05 for *F. ceranoides*, 0.01 for *F. spiralis* and 0.23 for *F. vesiculosus*). This analysis also showed that the two dioecious species *F. ceranoides* and *F. vesiculosus* were more closely related to one another than to the hermaphroditic *F. spiralis*, contradicting the results of Hardy et al. (1998), who concluded that *F. ceranoides* was the more distant taxon within this species complex. However, they based their study on the chemical phenotype of the taxa, which may be highly influenced by the estuarine environment of *F. ceranoides*.

Finally, the genetic distance between *F. vesiculosus* from estuary and open coast habitats (0.32) was twice as large as the genetic distance within the *F. vesiculosus* open coast group (0.15) and three times as large as that within the *F. vesiculosus* river group (0.09). This differentiation between rocky shore and estuarine *F. vesiculosus* might reflect restricted dispersal between estuarine and rocky shore populations, possibly due to geographic distance between locations or hydrodynamic factors. Local population acclimation or adaptation to specific habitats causing lower establishment success between habitats cannot be ruled out as an additional explanation for this rocky shore *vs.* estuarine population differentiation. The values of Nei genetic distances (*D*) observed between taxa are rather large in comparison with the empirically-defined threshold value of $D \geq 0.15$ that has been accepted in the literature to delineate animal species (see for review: Sites and Marshall 2004). Nevertheless, these large *D* values confirm that the three taxa constitute very different genetic entities.

We conclude that our genetic results clearly support the separation of *F. vesiculosus*, *F. spiralis* and *F. ceranoides* into distinct species within the Brittany region. The three species could be identified as three different genetic entities independent of geography. However, in agreement with recently published papers (Wallace et al. 2004, Engel et al. in press), we suggest that reproductive isolation may not be complete between *Fucus vesiculosus* and *F. spiralis*. In *F. vesiculosus*, genetic differentiation among habitats occurs as a secondary level of variation. As with morphological characters (see references in Introduction), *F. vesiculosus* was the most genetically variable of the three species. *Fucus* species appear to be a fascinating model algal group to study speciation processes, because contrasting patterns of hybridization have been detected: limited recent contact zone between *F. serratus* and *F. evanescens* (Coyer et al. 2002) *vs.* mosaic hybrid zones between *F. vesiculosus* and *F. spiralis* (Wallace et al. 2004, Engel et al. in press and this study). Moreover, the maintenance of morphological and genetic differences in the *F. vesiculosus* / *F. spiralis* group is paradoxical in the face of potential interspecific gene flow. Microsatellite loci, and in particular the diagnostic locus identified in this study, open new doors for future study of selective forces involved in the conservation of species integrity.

4.5 Acknowledgements

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With the analysis of *F. spiralis*, *F. vesiculosus* and *F. ceranoides* at the regional scale, we confirmed the genetic isolation of these three entities. However, we also observed that this isolation was not complete and that genetically intermediate individuals between *F. vesiculosus* and *F. spiralis* could be detected, even when populations were not in direct contact. As well, genetic results from Engel et al (2005) demonstrated the existence of introgression between these two species, suggesting not only the possibility of hybridization but also the possibility of back-crosses between hybrids and the two parental species despite their contrasting reproductive systems. How is it possible? Are the hybrids hermaphroditic or dioecious? What is their fertility? In order to answer these different questions and to better understand the evolution of sex in these algae, we studied their sexual phenotypes as wall as the sexual phenotypes of the hybrids and we quantified their respective fertilities.

Finally, we aimed at verifying if the theoretical models of the evolution of sex, like Charnov's theory (1982) fitted with brown algae.

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The results of this analysis are presented in the chapter 3. They have been published (article 3) in the *European Journal of Phycology* (2005)

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ARTICLE 3

Analysis of sexual phenotype and prezygotic fertility in natural populations of *Fucus spiralis, F. vesiculosus* (Fucaceae, Phaeophyceae) and in their putative hybrids

5 ANALYSIS OF SEXUAL PHENOTYPE AND PREZYGOTIC FERTILITY IN NATURAL POPULATIONS OF *FUCUS SPIRALIS*, *F. VESICULOSUS* (FUCACEAE, PHAEOPHYCEAE) AND IN THEIR PUTATIVE HYBRIDS

Billard, E. [1,2], Serrao, E.A. [2], Pearson, G.A. [2], Engel, C.R. [1], Destombe, C. [1] and Valero, M. [1] (2005) Analysis of sexual phenotype and prezygotic fertility in natural populations of Fucus spiralis, F-vesiculosus (Fucaceae, Phaeophyceae) and their putative hybrids. *European Journal of Phycology*, 40, 397-407.

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5.1 Abstract

In the genus *Fucus* the character dioecy/hermaphroditism has undergone multiple state changes and hybridization is possible between taxa with contrasting mating systems, e.g., between the dioecious *Fucus vesiculosus* and the hermaphroditic *F. spiralis*. In the context of mating system evolution, we evaluated the potential consequences of hybridization by studying the variation in sexual phenotype and in prezygotic fertility. First, as a result of hybridization between the two sexual systems, gender variation may arise depending on the relative importance of genes with large versus small phenotypic effects. We thus qualitatively examined the extent of gender variation within and among individual hybrids in comparison with both parental species. Second, if hybridization breaks up co-adapted gene complexes, hybrid fertility may be reduced in comparison with both parental species. We therefore also quantified male and female prezygotic fertility in parental species and their hybrids in order to test for reduction in hybrid fitness. A total of 89 sexually mature individuals (20 *F. spiralis*, 40 *F. vesiculosus* 10 hermaphroditic hybrids and 19 dioecious

hybrids) were sampled in two geographically distant regions (France and Portugal) and six conceptacles per individual were observed. Within-individual variation was very restricted qualitatively—only one hybrid carried a conceptacle with a different sexual phenotype from the five others—as well as quantitatively. This suggests a simple genetic system for sex determination involving a few genes with major effects. In addition, analyses showed no significant decrease in hybrid fertility compared with parental species. Moreover, hybrids exhibited all sexual phenotypes, suggesting several generations of hybridization and backcrossing and, therefore, that hybrids are reproductively successful. Finally, the occurrence of sterile paraphyses in female and hermaphroditic individuals was interpreted as a relic of male function and suggests that, as in higher plants, evolution from hermaphroditism to dioecy may be the most parsimonious pathway.

Keywords:

Dioecy, fucoid seaweed, hermaphroditism, hybridization, male and female function, mating system evolution, sexual allocation, sperm/egg ratio

5.2 Introduction

Studying variation in reproductive strategies in hybridizing taxa provides an excellent opportunity for directly studying the factors that influence the evolutionary pathway between different reproductive systems (Barrett, 1998; Charlesworth, 1999; Hewitt, 2001; Dorken *et al.*, 2002; Dorken & Barrett, 2003). While changes in the mating system can influence the degree of reproductive isolation of the parental species, reproductive isolation may also evolve due to divergent selective pressures in different habitats (e.g., Sakai & Weller, 1999; Barrett *et al.*, 2001; Dorken & Barrett, 2003). In stable, highly stressful habitats, selfing may present a real advantage compared to outcrossing (for review, see Takebayashi & Morrell, 2001), by (1) increasing reproductive assurance and (2) maintaining local adaptation (e.g., via co-adapted gene complexes). On the other hand, in habitats where competition is strong, outcrossing may limit inbreeding depression. Differences in mating system may sometimes operate as a genetic barrier maintaining the two species in the two different environments.

Fucus, a genus of ecologically successful brown seaweeds, is one of the rare groups where hybridization in natural populations has been reported between taxa possessing contrasting mating systems. Hybridization between cosexual hermaphroditic and unisexual dioecious species has been suggested by the observation of intermediate thallus morphologies in the field between cosexual *F. spiralis* and unisexual *F. ceranoides* (Gard, 1910), as well as field observations and/or laboratory crosses between *F. spiralis* and unisexual *F. vesiculosus* (Sauvageau, 1909; Kniep, 1925; Burrows & Lodge, 1951; Scott & Hardy, 1994); between cosexual *F. evanescens* and unisexual *F. serratus* (Lein, 1984, Rice & Chapman, 1985 ; Coyer *et al.*, 2002) and has been recently confirmed in natural populations using molecular markers for the latter two species pairs (Coyer *et al.*, 2002; Wallace *et al*., 2004, Engel *et al.*, 2005).

Vernet & Harper (1980) advanced the hypothesis of contrasting selective pressures—abiotic on the upper shore and biotic on the lower shore—driving divergence in mating systems in *F. spiralis* and *F. vesiculosus*, which co-occur throughout most of their distributional range. Typically, the vertical distribution of the species is discontinuous (parapatric situation) where hermaphroditic *F. spiralis* is present in the high intertidal zone and dioecious *F. vesiculosus* in the mid-intertidal zone. However, individuals of the two species can be found in contiguous stands (sympatric situation) in the transition zone and

on shores with complex topology. Due to their overlapping but distinct vertical distributions on the shore, on average *F. spiralis* individuals are emersed at low tide longer than *F. vesiculosus* individuals, and are therefore subjected to different selective pressures in response to abiotic stress. Although laboratory studies of emersion stress reveal little difference in tolerance between *F. spiralis* and *F. vesiculosus* (e.g., Dring & Brown, 1982), field studies suggest that (sublethal) emersion stress mediates competitive interactions (perhaps between early post-settlement or juvenile stages). For example, it has been shown that *F. vesiculosus* can extend its vertical range upshore when *F. spiralis* is removed (Hawkins & Hartnoll, 1985; Chapman & Johnson, 1990); however, *F. spiralis* is competitively excluded by *F. vesiculosus* in the midshore region (Chapman, 1990; see reviews by Chapman, 1995; Davison & Pearson, 1996).

In *Fucus*, the character dioecy/hermaphroditism has undergone multiple state changes (Serrão *et al.*, 1999). Although the taxa sister to *Fucus* are hermaphroditic, the scattered phylogenetic positions of hermaphroditic and dioecious species suggests that either mating system changed independently several times, possibly by relatively simple mechanisms (Serrão *et al.*, 1999) as in the flowering plant genus *Silene* (Desfeux *et al.*, 1996), and in angiosperms generally (for review see Charlesworth, 2002). In land plants, based on modelling, empirical and phylogenetic studies, dioecy generally appears to be the derived state arising from cosexual ancestors (Charlesworth, 1999). In many dioecious species for which hermaphrodite relatives are known, evidence for gender instability (i.e. sexual variation among flowers within the same individual) and in particular rudiments of structures of the opposite sex in flower morphology, were first observed by Darwin (1877) and provide further support for this evolutionary pathway (Webb, 1999; Charlesworth, 2002).

In *Fucus*, reproductive organs are grouped within conceptacles borne on fertile structures (receptacles); by analogy with flowering plants, conceptacles are cosexual in hermaphroditic species but unisexual (male or female) in dioecious species. Male gametes are enclosed in antheridia borne by branched filaments with chromatophores, called paraphyses, whereas female gametes are enclosed in oogonia (reviewed by Fritsch, 1945, p. 368 and illustrations). Fritsch also observed that paraphyses were present in female conceptacles although less ramified than the paraphyses in male conceptacles. Moreover, in this group, the sexual phenotype has been described as one of the most stable characters of a genus that otherwise shows a high degree of vegetative morphological plasticity (Burrows & Lodge, 1951; Pérez-Ruzafa *et al.*, 1993; Pérez-Ruzafa, 2001). The only reported gender

instability in this genus concerns subdioecious individuals (with both cosexual and unisexual conceptacles) that were observed in typically unisexual *F. ceranoides* (Thuret & Bornet 1878; Baker & Bohling 1916, cited in Pérez-Ruzafa, 2001), although this has also been cited as evidence of hybridization with *F. spiralis* (Gard, 1910; Lein, 1984). However, the extent of gender instability within individuals has never been quantified at the population level. Moreover, as a result of hybridization between the two sexual systems, theoretical studies by Charlesworth & Charlesworth (1978a, 1978b) show that gender variation among conceptacles may be discrete or continuous depending on whether a few genes with large, or many genes with small, phenotypic effects control sexual phenotype. Hybrids may thus give rise to qualitatively and/or quantitatively intermediate sexual phenotypes. In addition, as hybridization may break up co-adapted gene complexes, hybrid fertility may be reduced in comparison with either parental species.

In this paper, we first examined the extent of gender variation within and among individual hybrids in comparison with both *F. vesiculosus* and *F. spiralis* species. Secondly we quantified male and female prezygotic fertility in parental species and their hybrids in order to test for reduction in hybrid fitness.

5.3 Materials and Methods

5.3.1 Sampling

To compare hybrids with their parental species, we used individuals previously characterized by Engel *et al.* (2005) for their sexual phenotype (hermaphrodite *vs*. dioecious) and genetic status (hybrid vs. parental species). In the aforementioned study, the sexual phenotype of each individual was established by qualitative observation of a single receptacle per individual. In parallel, individuals were genotyped for five microsatellite loci developed by Engel *et al.* (2003) and the occurrence of genetically intermediate individuals was determined on the basis of genotypic assignment tests, using the software package STRUCTURE (Pritchard *et al.*, 2000) where the proportion of each species' genome was estimated for each individual. Individuals for which the proportion of one of the genomes was between 0.1 and 0.9 were considered as putative hybrids (Engel *et al.*, 2005 Fig. 3).

In the present study, a total of 89 sexually mature individuals were selected [20 hermaphroditic *F. spiralis* (Fs), 10 hermaphroditic individuals classified as hybrids, 40 dioecious *F. vesiculosus* (Fv) (20 males and 20 females) and 19 dioecious hybrids (10

males and 9 females)] for a quantitative estimation of their fertility. Individuals were sampled from two types of situations: one called "parapatric", where the two species' habitats were separated on the shore; and one called "sympatric", where both taxa were found in contiguous stands. The same sampling was repeated in two geographically distant regions, at Cape Gris-Nez in the North of France and at Viana do Castelo in the North of Portugal. The details of the sample size for each taxon and sexual phenotype within each region are shown in Table 1.

Table 1: Means and standard errors (SE) for individual male and female fertilities calculated over the different taxa and population types.

a. Male fertility[1]

Taxon	Mean ± SE	Region	Situation	n	Mean ± S-E
Male *F. vesiculosus*	1677 ± 42	Gris Nez	Parapatric	5	1548 ± 63
		Viana	Parapatric	5	1727 ± 81
		Gris Nez	Sympatric	5	1831 ± 116
		Viana	Sympatric	5	1601 ± 51
Hermaphrodite *F. spiralis*	162 ± 13	Gris Nez	Parapatric	5	130 ± 14
		Viana	Parapatric	5	216 ± 40
		Gris Nez	Sympatric	5	127 ± 13
		Viana	Sympatric	5	175 ± 21
Male hybrids	1899 ± 99	Gris-Nez		5	1409 ± 110
		Viana		5	2388 ± 108
Hermaphrodite hybrids	119 ± 13	Gris-Nez		5	86 ± 10
		Viana		5	151 ± 22

b. Female fertility[2]

Taxon	Mean ± S-E	Region	Situation	n	Mean ± S-E
Female *F. vesiculosus*	47.32 ± 1.73	Gris Nez	Parapatric	5	36.54 ± 2.95
		Viana	Parapatric	5	57.85 ± 3.47
		Gris Nez	Sympatric	5	47.40 ± 3.15
		Viana	Sympatric	5	47.48 ± 3.24
Hermaphrodite *F. spiralis*	36.41 ± 2.16	Gris Nez	Parapatric	5	31.02 ± 2.39
		Viana	Parapatric	5	23.35 ± 1.33
		Gris Nez	Sympatric	5	48.27 ± 6.75
		Viana	Sympatric	5	42.99 ± 3.22
Female hybrids	35.01 ± 1.95	Gris-Nez		4	33.79 ± 3.12
		Viana		5	35.99 ± 2.52
Hermaphrodite hybrids	30.73 ± 1.97	Gris-Nez		5	22.06 ± 1.81
		Viana		5	39.41 ± 2.71

n: number of sampled individuals.
[1]: density of antheridia per conceptacle (number of antheridia / mm^2 conceptacle section)
[2]: density of oogonia per conceptacle (number of oogonia / mm^2 conceptacle section)

5.3.2 Variation in sexual phenotype and male and female fertility within individuals

For each parental taxon and their hybrids, sexual phenotype was characterized for six conceptacles (i.e. three in each of two receptacles) per individual. To search for sexual

inconstancy (i.e. variation in gender within individuals), we investigated the extent of variation in male and female fertility within individuals. For each of the 89 selected individuals, two freeze-dried receptacles were re-hydrated in seawater for 10 minutes, then 60 µm frozen sections were made with a cryo-microtome, in the mid-basal part of the receptacle to standardize measurements between conceptacles. Three conceptacles per receptacle were observed under a microscope with Spot Basic Software (Diagnostic Instruments Inc., 1996-2003). Female fertility was estimated as the density of oogonia per section of conceptacle (number of oogonia / mm^2 conceptacle section) and male fertility as the density of antheridia per section of conceptacle (number of antheridia / mm^2 conceptacle section). In contrast to hermaphroditic individuals, it was not feasible to count all antheridia in sections of unisexual male conceptacles; consequently, we used an estimate of this number (mean density of antheridia over three different unitary areas). The intra- and inter-individual components of fertility variation were quantified for both parental taxa and their hybrids. Since sympatric situations tended to experience more genetic admixture (Engel *et al.*, 2005), the analyses were carried out separately for parapatric and sympatric situations. Within these different situations, the proportional contribution of intra-individual and inter-individual variance to the overall variation in male and female fertilities was estimated using a nested model ANOVA

$$Y_{ijn} = \mu + \alpha_i + \beta_{ij} + e_{ijn}$$

where Y_{ijn} is the fertility of the n^{th} conceptacle of receptacle j nested in the i^{th} individual, μ is the individual mean, α_i is the random contribution of the i^{th} individual, β_{ij} is the random contribution of the j^{th} receptacle of the i^{th} individual and e_{ijn} is the error term. In order to obtain a rough estimate of the relative contribution of within individual variation to the overall variance, we added receptacle and conceptacle (i.e., error term) variances.

5.3.3 Variation for male and female fertilities between both parental taxa

Prior to testing for fertility reduction in hybrids, the pattern of variation in male and female fertilities was evaluated within and between the two parental species in order to investigate the effect of environmental variation (i.e. difference between regions) on sexual allocation

to male and female functions. Moreover, species differences may be reinforced in sympatric compared to parapatric situations (Servedio & Noor, 2003). Male and female individual fertilities (i.e. fertilities averaged over individuals) were compared between species, distributional situations (parapatric *vs* sympatric) and regions (Gris Nez *vs* Viana) using the following mixed-model ANOVA:

$$Y_{ijkn} = \mu + A_i + B_j + \delta_k + (AB)_{ij} + (A\delta)_{ik} + (B\delta)_{jk} + (AB\delta)_{ijk} + e_{ijkn}$$

where Y_{ijkn} is the fertility of the n^{th} individual of the i^{th} species, belonging to the j^{th} population type and sampled in the k^{th} region. μ is the species mean, A_i is the fixed effect of the i^{th} species, B_j is the fixed effect of the j^{th} population type, δ_k is the random effect of the k^{th} region. $(AB)_{ij}$, $(A\delta)_{ik}$ and $(B\delta)_{jk}$ are respectively the interaction effect of 'species x population type', 'species x region' and 'population type x region'. $(AB\delta)_{ijk}$ is the 'species x population type x region' interaction effect and e_{ijkn} is the error term.

5.3.4 Comparison between hybrids and parental species

The putative reduction of male or female individual fertility in hybrids compared to parental species was tested using the following mixed-model ANOVA.

$$Y_{ijn} = \mu + A_i + \beta_j + (A\beta)_{ij} + e_{ijn}$$

Where Y_{ijn} is the fertility of the n^{th} individual of the i^{th} taxon (parental *vs* hybrid). μ is the taxon mean, A_i is the fixed effect of the i^{th} taxon; β_j is the random effect of region $(A\beta)_{ij}$ is the interaction effect between taxon and region and e_{ijn} is the error term.

Finally, to summarise the information for both male and female fitness components, the pattern of sex allocation in male and female functions estimated as sperm/egg ratio (S/E) was compared between hermaphrodites and dioecious taxa. For hermaphrodites this ratio was obtained for each individual by multiplying the number of antheridia in the section by 64 (number of sperm per antheridium) and the number of oogonia in the section by 8 (number of eggs (ovule) per oogonium). For the dioecious taxa, an approximation was calculated using the total number of sperm and the total number of ovules produced in each situation in each region, following the suggestion of Vernet & Harper (1980). In addition,

for the 10 hermaphroditic hybrid individuals, the evolution of S/E ratio was reported according to the proportion of *F. vesiculosus* genome estimated from the genotype data of Engel *et al.*, (2005). The effect of the dioecious species genotypes on the pattern of sex allocation was tested using Spearman rank correlation.

All ANOVAs were conducted using the general linear model procedure of MINITAB (version 13.2 MiniTab Inc. 1994, State College USA). Male fertility (density of antheridia) and female fertility (density of oogonia) were log-transformed in order to meet the normality and homoscedasticity requirement of ANOVA. Multiple comparisons of means were performed using the GT2 method recommended for unequal sample sizes (Sokal & Rohlf, 1995, p. 244)

5.4 Results

Figure 1a: Male fertility

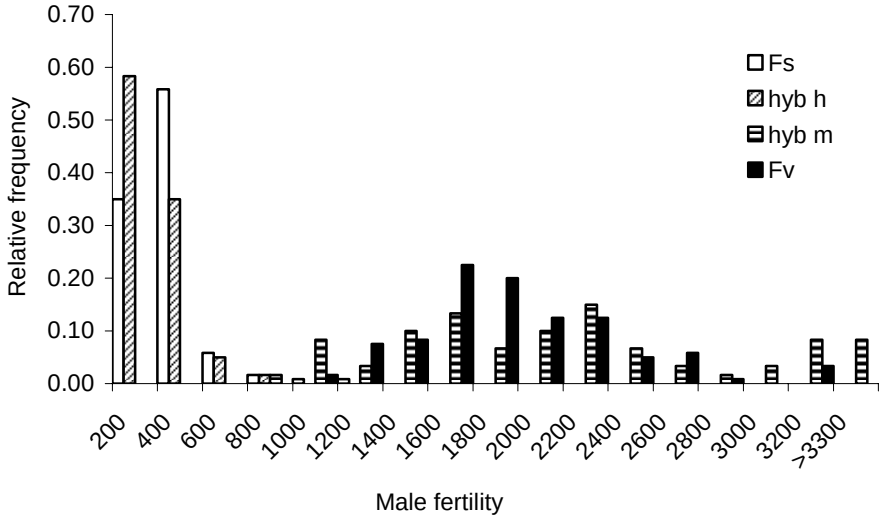

Fig. 1b: Female fertility

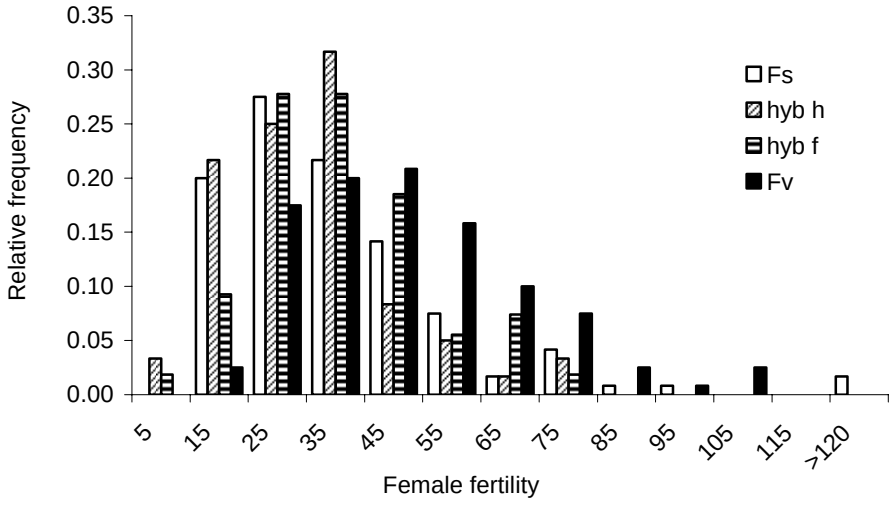

Figure 1. Frequency distribution of (a) male fertility[1] and (b) female fertility[1] estimated from the 534 conceptacles from hermaphroditic individuals (Fs: *F. spiralis* and hyb h: hermaphroditic hybrids) and dioecious individuals (Fv: *F. vesiculosus*, hyb m: male hybrids and hyb f: female hybrids).
[1] See Table 1 legend for definition of these variables

While male conceptacles were easily recognizable, it was more difficult to distinguish between female and hermaphroditic conceptacles because of the occurrence of numerous sterile paraphyses in many cross sections. Since sterile paraphyses appear to represent aborted male structures, their occurrence was recorded for each conceptacle. Sterile filaments were never observed in male individuals; on the other hand, they were present in at least one out of the six sampled conceptacles per individual in females and hermaphrodites.

The frequency distribution of male and female fertilities estimated from the 534 observed conceptacles is given in Figure 1. The distribution of male fertility was clearly multimodal and discriminated cosexual from unisexual individuals (Fig. 1a). The density of antheridia varied from 0 to 973 antheridia per mm^2 conceptacle section area for *F. spiralis* and hermaphroditic hybrids, and from 691 to 3920 in *F. vesiculosus* and unisexual hybrids. Consequently, the mean male fertility of both hermaphrodite taxa was ten times smaller than in dioecious taxa (Table 1a). This difference in fertility between unisexual and cosexual phenotypes was not found for the female function. The distribution of female fertility appeared unimodal and overlapped between the four taxa (Fig. 1b). The density of oogonia varied greatly among conceptacles (from 4 to 168 in hermaphrodites and from 5 to 103 in dioecious taxa). Inspection of mean values suggested that female fertility tended to be lower in hermaphroditic taxa (Table 1b), although the effect seems to depend on the region and distributional situation (see below).

5.4.1 Variation of sexual phenotype within individuals

Among the 89 individuals studied, variation in sexual phenotype among conceptacles within an individual was only observed in the single case of a hermaphroditic hybrid individual that exhibited one male-sterile (i.e. female) conceptacle while the other five conceptacles were clearly hermaphroditic. The only other qualitative patterns of sex inconstancy revealed concerned the presence/absence of sterile paraphyses in hermaphroditic and female individuals. This inconstancy was observed in both parental species and hybrids.

Table 2. Result of nested ANOVA on the relative contribution (%) of between *versus* within-individual effects on the variance of male and female fertility, analyzed separately for parapatric and sympatric situations in each of the two parental species and in unisexual and hermaphroditic hybrids.

a. Male fertility[1]

Group	Between-individual effect		Within–individual (receptacle) effect		error
	%	F	%	F	%
Male *Fv* Parapatric	6.81	1.48	0†	0.91	93.19
Male *Fv* Sympatric	48.46	4.41*	16.86	2.46*	34.68
Hermaphroditic *Fs* Parapatric	28.97	4.27*	0†	0.75	71.03
Hermaphroditic *Fs* Sympatric	39.95	3.85*	12.04	1.75	48.01
Male hybrids	71.26	11.23 ***	6.54	1.88	22.20
Hermaphroditic hybrids	30.85	3.56*	0.84	1.04	68.31

b. Female fertility[1]

Group	Between-individual effect		Within–individual (receptacle) effect		error
	%	F	%	F	%
Female *Fv* Parapatric	52.51	8.58 **	0†	0.87	47.49
Female *Fv* Sympatric	44.74	4.16 *	14.83	2.10*	40.43
Hermaphroditic *Fs* Parapatric	36.52	3.59 *	10.63	1.60	52.85
Hermaphroditic *Fs* Sympatric	36.94	2.79	30.36	3.78**	32.70
Female hybrids	10.5	0.51	16.63	1.68	72.86
Hermaphroditic hybrids	58.26	6.91**	8.72	1.79	33.02

Fv = F. vesiculosus, *Fs = F. spiralis*

Significant F values: *P <0.05 ; ** P < 0.01 ; *** P < 0.001

†: Negative values close to zero due to larger within than among group variance.

[1] See Table 1 legend for definition of these variables

5.4.2 Variation in male and female fertility within individuals

Quantitative variation in sexual phenotype (i.e. variation in male or female fertility) was generally not significant among receptacles, whereas it was significant among individuals (see nested ANOVA, Table 2), indicating that within-individual variation was less important than variation among-individuals. Over the 12 ANOVAs, variation among receptacles was significant in only three cases whereas variation between individuals was significant in all but three cases (Table 2). However, even when non-significant values are removed, the proportion of total variation explained by inter-individual effects varied greatly among taxa (from 29% to more than 70%, Table 2) revealing some weak but interesting patterns, summarized below.

Within-individual variance of male fertility tended to be larger in sympatric than in parapatric situations in the two parental species (Table 2a). However, no increase in within-individual variation was detected in hybrids in comparison with either parental species. Indeed, the lowest contribution of within-individual variance to the overall (within plus between) variance of male fertility was observed in male hybrids. Finally, significant within-individual variation was only observed in dioecious species (significant contribution of receptacles in sympatric situations, but not in parapatric situations, Table 2a).

In contrast to male fertility, within-individual variance of female fertility tended to be larger in the hermaphroditic species. Indeed, in sympatric situations, the contribution of the within-individual term obscured the contribution of the between-individual term (Table 2b). In hybrids, contrasting results were obtained depending on the sexual phenotype. In female hybrids, no significant variation among individuals was observed while inter-individual variation was highly significant in hermaphroditic hybrids, explaining more than 50% of the overall variance in female fertility (Table 2b).

5.4.3 Variation in male and female fertility between parental taxa

The most obvious difference between parental species concerned the pattern of sex allocation between male and female functions. The density of antheridia of *F. vesiculosus* was much higher than in *F. spiralis* (Table 1a and Table 3a). Although there was a trend for *F. vesiculosus* to have greater densities of oogonia than *F. spiralis*, it was not consistently significant across all situations and regions (Table 3b, see also Fig. 1b and Table 1b). The much larger difference in male than in female fertility meant that the two species were easily distinguishable by their mean Sperm/Egg ratio values (S/E = 385.4 ± 35.8 for *F. vesiculosus*, and S/E = 44.3 ± 6.1 for *F. spiralis*).

5.4.4 Comparison between hybrids and parental species

When comparing hybrids with *F. vesiculosus* or *F. spiralis* for male (Table 4a) and female (Table 4b) functions, there was no significant taxon effect. However, in two of the four cases, the divergence between parental taxa and hybrids varied depending on the region (cf. significant Taxon*Region interaction, Table 4). Hybrid fertility was lower than parental fertility in Gris-Nez but higher in Viana. Hybrid male fertility was higher than *F. vesiculosus* male fertility in Viana (GT2 multiple comparison of means tests, $p<0.05$). The same trend is observed in Viana with female fertility higher in hermaphroditic hybrids than

in *F. spiralis,* however multiple comparisons were inconclusive. Therefore, there was no overall trend for hybrid superiority or inferiority.

Table 3. Results of mixed model ANOVAs on effects of species, distributional situation (parapatric / sympatric) and region (Gris-Nez / Viana), on male (a) and female fertility (b).

a. Male fertility [1]

Source	df	MS	F	P
Species	1	12.3639	273.30	0.038
Distributional situation	1	0.0001	0.01	0.943
Region	1	0.0476	0.84	0.484
Species x Distributional situation	1	0.0043	8.63	0.209
Species x Region	1	0.0452	91.74	0.066
Distributional situation x Region	1	0.0117	23.67	0.129
Species x Distributional situation x Region	1	0.0005	0.02	0.894
Error	32	0.0274		
Total	39			

b. Female fertility [1]

Source	df	MS	F	P
Species	1	0.1945	2.86	0.340
Distributional situation	1	0.1140	17.58	0.149
Region	1	0.0085	0.84	0.968
Species x Distributional situation	1	0.0800	1.24	0.466
Species x Region	1	0.0680	1.06	0.491
Distributional situation x Region	1	0.0065	0.10	0.804
Species x Distributional situation x Region	1	0.0645	3.43	0.073
Error	32	0.0188		
Total	39			

[1] See Table 1 legend for definition of these variables

Table 4. Results of mixed model ANOVAs comparing male and female fertility between (a) *F. vesiculosus* and unisexual hybrids and (b) *F. spiralis* and hermaphroditic hybrids.

	F. vesiculosus vs. unisexual hybrids				*F. spiralis* vs. hermaphroditic hybrids			
a. Male fertility[1]								
Source	df	MS	F	P	df	MS	F	P
Taxon	1	0.0072	0.07	0.834	1	0.1014	42.97	0.096
Region	1	0.1040	0.03	0.496	1	0.1603	67.94	0.077
Taxon*Region	1	0.1011	12.49	0.002	1	0.0024	0.06	0.814
Error	26	0.0081			26	0.0420		
Total	29				29			
b. Female fertility[1]								
Source	df	MS	F	P	df	MS	F	P
Taxon	1	0.1193	11.51	0.182	1	0.0317	0.17	0.748
Region	1	0.0306	2.96	0.335	1	0.0830	0.46	0.621
Taxon *Region	1	0.0104	0.64	0.430	1	0.1813	6.42	0.018
Error	25	0.0161			26	0.0282		
Total	28				29			

[1] See Table 1 legend for definition of these variables

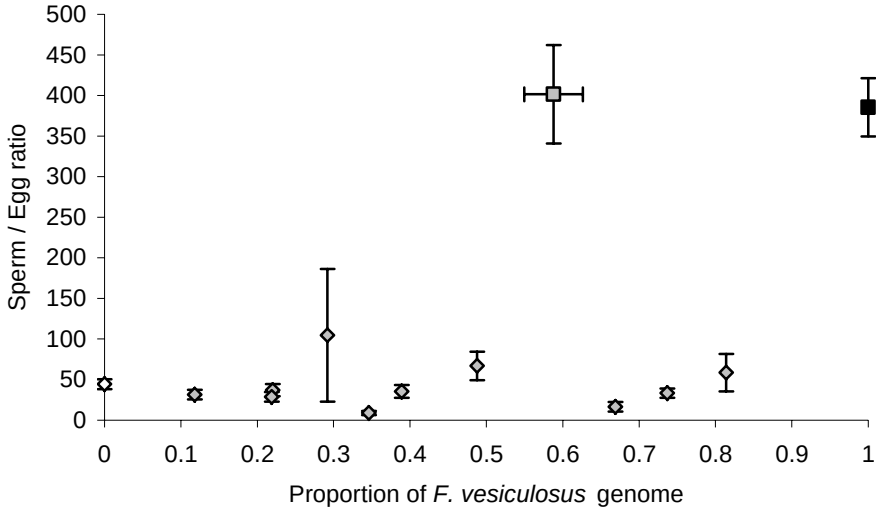

Figure 2. Sperm/Egg (S/E) ratios of hermaphroditic hybrids are represented (grey diamonds) according to genetic admixture (i.e. proportion of genome characteristic of *F. vesiculosus*) (see Engel *et al.*, 2005). The open diamond is the mean S/E ratio for *F. spiralis*, and the closed square is the mean S/E ratio for *F. vesiculosus*. The mean S/E ratio according to the mean genetic admixture of dioecious hybrids is also presented (grey square), with x and y standard error bars. Diamonds: hermaphroditic individuals; squares: dioecious individuals.

Finally, the mean Sperm/Egg ratios calculated for unisexual and cosexual hybrids were very similar to the corresponding parental sexual phenotype (i.e 401.4(± 60.6) for dioecious hybrids *vs* 385.4 (± 35.8) for *F. vesiculosus*; and 42.2(± 8.8) for hermaphroditic hybrids *vs* 44.3 (± 6.1) for *F. spiralis*). The S/E ratio of hermaphroditic hybrids is presented in Figure 2. The S/E ratio of hermaphroditic hybrids was not correlated with the proportion of *F. vesiculosus*' genome (Spearman Rank correlation coefficient = 0.0009). Whatever the proportion of *F. vesiculosus* genome in hermaphroditic hybrids, their S/E ratio was always close to the S/E ratio of *F. spiralis*.

5.5 Discussion

Our results verify that sexual phenotype is a stable character within individuals of these two species. It was further shown that no quantitatively intermediate sexual phenotypes were present in hybrids. Together, this suggests that the genetic basis of sex determination in *Fucus* is controlled by a few genes with large effects. The second main

result was that there were no differences in prezygotic fitness-related parameters (male and female gametangia densities) between putative hybrids and parental taxa.

The general absence of gender variation among conceptacles suggests the occurrence of a strong genetic component in sex determination. In brown seaweeds, a single Mendelian determinant of sex was first demonstrated in *Ectocarpus* spp. (Müller, 1967). The occurrence of sex chromosomes has been proposed in Laminariales (Evans, 1963; Fang, 1983) and in fucoids (Clayton, 1984), but has not yet been proved.

Among the 58 putative hybrids detected (see Engel *et al.*, 2005), all three sexual phenotypes were present: males (43%), females (28%) and hermaphrodites (28%). For only one individual was sexual phenotype not determined, probably due to immaturity. It has been reported (Westergaard, 1958) that when monoecious or hermaphroditic and dioecious species are crossed, it is very rare to observe all sexual phenotypes in the first generation of hybrids (F1) (see Appendix); furthermore, it is rare for all these sexual phenotypes to be fertile. Thus, it is possible that some sexual phenotypes are the result of second-generation hybrids (F2) and/or backcrosses. This implies that *F. spiralis* x *F. vesiculosus* hybrids are capable of backcrossing (suggested by Engel *et al.*, 2005), possibly due to the extremely recent divergence between *F. vesiculosus* and *F. spiralis* (Serrão *et al.*, 1999). Moreover, if we consider that the most probable crosses involve male individuals rather than hermaphroditic ones (see Appendix), the observed proportions of the three genders are consistent with sex determination with male heterogamety. However, these results are in contradiction with those of Coyer *et al.* (2002) who observed only unisexual hybrids between *F. serratus* and *F. evanescens*. No simple sex determination system exists to explain this difference, except a nucleo-cytoplasmic sex determination

In this study we had no means to determine the generation of hybrids; this would require that crosses be performed under controlled conditions. However, the occurrence of backcrosses or F2 crosses is also a hypothesis that is supported by the lack of differences in fertility between hybrids and parental species. This means that hybrids are potentially as fertile as their parents. Our analyses of hybrid (prezygotic) fitness suggest that this may be greater or less than that of parental taxa, depending on region-specific effects. Indeed, it has been suggested (see Arnold, 1997) that both endogenous (e.g., disruption of co-adapted gene complexes) and environment-dependent selection on hybrids may operate. An example of the latter are results from hybrid zones of two *Quercus* species (Williams *et al.*, 2001), in which hybrids were not necessarily less fit than their parents, especially in marginal areas. Indeed, our results show that hybrid fertility may be higher than parental

fertility in Viana, the southern limit of co-occurrence of both *F. spiralis* and *F. vesiculosus* species. It would be interesting to extend these results by investigating the relationship between relative hybrid fitness and environmental factors (e.g., those associated with geographic range).

The relative male and female fitness components in hybrids can be summarized by examining the sperm/egg (S/E) ratios for hermaphrodite and dioecious taxa. Again, for the same sexual phenotype (i.e., mating system), there were no differences in S/E between parental taxa and hybrids, while there was one order of magnitude difference between mating systems. These S/E ratios, obtained from cross-sections of conceptacles, were very similar to those obtained by Vernet & Harper, (1980) on whole conceptacles (respectively 400 and 40). It is interesting to compare these S/E ratios of *F. vesiculosus*, *F. spiralis* and hybrids with the mean Pollen/Ovule ratios (P/O) characteristic of different breeding systems in angiosperms (Cruden, 1977). Indeed, P/O ratios increase significantly with the likelihood of cross-fertilization and are therefore a good indicator of the breeding system. The hermaphroditic taxa, with a mean S/E ratio of 43, correspond to obligate and facultative self-fertilization, which is consistent with population genetic data revealing high heterozygote deficiencies in *F. spiralis* (Engel *et al.*, 2005). On the other hand, dioecious taxa, with ratios of 400 correspond to the classes of facultative self-fertilization and facultative cross-fertilization. Since selfing is impossible in dioecious taxa, these ratios indicate biparental inbreeding; this hypothesis is also supported by significant heterozygote deficiencies revealed by population genetic data (Engel *et al.* 2005).

Interestingly, we found variation in the presence or absence of sterile paraphyses; In this study, sterile paraphyses were present in at least one conceptacle per individual in females and hermaphrodites but absent in males, where all observed paraphyses bore antheridia. The occurrence of sterile paraphyses is very interesting in the context of mating system evolution since they appear to correspond to relics of the antheridium-bearing paraphyses, which are present in conceptacles with male function. This is similar to land plants, where unisexual individuals of many dioecious species with cosexual relatives bear evident relics of the opposite sex (Darwin, 1877). In this context, hermaphroditism is considered as the primitive state (see for review Geber *et al.*, 1999). Several evolutionary scenarios can explain the evolution of dioecy from cosexuality (Geber *et al.*, 1999); one involving a minimum of two major mutations (one causing male sterility and the other female sterility), while another involves many mutations with smaller effects and a range of intermediate sexual phenotypes (Charlesworth, 2002).

The Fucaceae show a wide range of variation of mating systems among its species: hermaphroditic (most genera), monoecious (only the genus *Xiphophora*, having separate male and female conceptacles in the same individuals with occasional hermaphroditic conceptacles) and dioecious (*Ascophyllum nodosum* and some species of *Fucus*) systems are found. However, very little intra-specific variation has ever been reported in the Fucaceae (e.g., monoecious individuals have been reported in the dioecious species *F. ceranoides* (Hamel, 1939) although this is controversial as monoecious individuals may be result of hybridization between *F. ceranoides* and *F. spiralis* (Lein, 1984). Within the Sargassaceae, a sister group of the Fucaceae (Rousseau *et al.*, 2001), variation for mating system between and within species is even higher. For instance, in *Turbinaria ornata* one type of receptacle carries male or hermaphroditic conceptacles and the other type of receptacle carries female conceptacles; individuals may harbour only one type of receptacle or both and may thus be andromonoecious, female or polygamomonoecious (Stiger, 1997). The genus *Cystoseira*, which is mainly composed of hermaphroditic species, also includes some species whose individuals carry male, female and hermaphroditic conceptacles in the same receptacles (Hamel, 1939). Finally, in the genus *Sargassum*, some individuals of a monoecious species (*S. flavifolium*) harbour receptacles with male and female conceptacles whereas individuals of another species (*S. trichocarpum*) carry strictly male or female receptacles. In this context, Clayton (1984) speculated that in the order Fucales, the ancestral states could have been hermaphroditic, as in angiosperms. In this study, the observation of sterile filaments in females and the rarity of intermediate phenotypes in *F. vesiculosus* and *F. spiralis*, even among hybrids, support the hypothesis of a simple genetic sexual determination with few major genes involved and thus of evolution from hermaphroditism to dioecy via the gynodioecy pathway (Charlesworth, 1999; Charlesworth, 2002).

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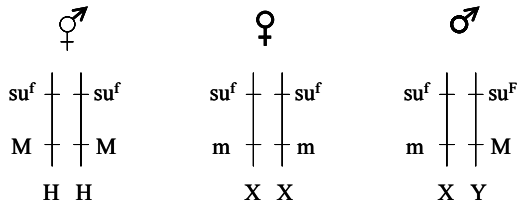
Appendix

1. Sex determination with male heterogamety

Su^F : Female fertility suppressor, M : Male fertility

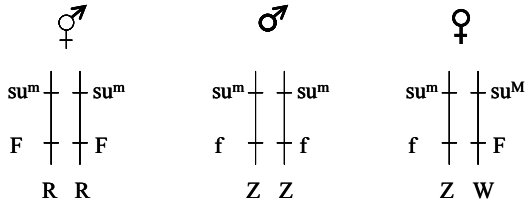


All possible F_1 crosses	**progeny**
hermaphrodite (HH) x male (XY) *****	50% hermaphrodite (HX)
	50% male (HY)
female (XX) x hermaphrodite (HH)	100% hermaphrodite (HX)
All possible back-crosses	
hermaphrodite (HX) x male (XY) *	50% male (HY + XY)
	25% hermaphrodite (HX)
	25% female (XX)
male (HY) x hermaphrodite (HH) *	50% hermaphrodite (HH)
	50% male (HY)
male (HY) x female (XX) *	50% male (XY)
	50% female (HX)
hermaphrodite (HX) x female (XX)	50% hermaphrodite (HX)
	50% female (XX)
hermaphrodite (HX) x hermaphrodite (HH)	100% hermaphrodite (HX +HH)

- The most probable crosses, due to the fact that male individuals produce ten times more sperm than hermaphroditic and thus that female and hermaphroditic individuals are more prone to be fertilized by a male than by a hermaphrodite. This crosses result in at least 50% of male progeny.

2. Sex determination with female heterogamety

Su^M : Male fertility suppressor, F : Female fertility



All possible F_1 crosses	**progeny**
hermaphrodite (RR) x males (ZZ) *	100% hermaphrodite (RZ)
female (ZW) x hermaphrodite (RR)	50% hermaphrodite (RZ)
	50% female (RW)
All possible back-crosses	
hermaphrodite (RZ) x male (ZZ) *	50% hermaphrodite (RZ)
	50% male (ZZ)
female (RW) x male (ZZ) *	50% female (ZW)
	50% hermaphrodite (RZ)
hermaphrodite (RZ) x female (ZW)	50% female (RW + ZW)
	25% hermaphrodite (RZ)
	25% male (ZZ)
female (RW) x hermaphrodite (RR)	50% hermaphrodite (RR)
	50% female (RW)
hermaphrodite (RZ) x hermaphrodite (RR)	100% hermaphrodite (RZ +RR)

* The most probable crosses, due to the fact that male individuals produce ten times more sperm than hermaphroditic and thus that female and hermaphroditic individuals are more prone to be fertilized by a male than by a hermaphrodite. This crosses result in an excess of hermaphroditic progeny.

The study of sexual phenotypes of hybrids between *F. vesiculosus* and *F. spiralis* enabled to show that hybrids possess the same reproductive characteristics as the parental species, without fertility decrease. Firstly, this suggests that sex determination must be under the control of a (major) genetic factor rather than of environmental factors; secondly, it confirms the hypothesis of introgression between species.

On the other hand, results obtained by the analysis of resource allocation to sexual functions indicate that *F. spiralis*, which is found in the upper part of the shore, tends to reproduce by selfing while *F. vesiculosus*, although obligate out-crosser (dioecious species), presents important heterozygote deficits. It seems thus that male and gamete dispersion is relatively spatially restricted, potentially limiting hybridization and therefore maintaining the relative integrity of both species in natural populations.

To verify this hypothesis, we tried to estimate gamete dispersal on the shore, by studying the gene flows (within and between species) in the transition zone from one species to another. Results are presented in chapter 4, in which the extent and the orientation of putative hybridization were estimated on one hand with new microsatellite markers specially developed for *F. spiralis* (Perrin et al., 2007) in addition to the microsatellite markers used in chapter 2 and on the other hand by the use of two chloroplastic markers, allowing to follow female gene flow.

This analysis gives rise to the Article 4 (in prep)

ARTICLE 4

A mosaic of hybrids between species with contrasting reproductive systems at the micro-spatial-scale of the shore: phenotypic and genetic analyses.

6 A MOSAIC OF HYBRIDS BETWEEN SPECIES WITH ACONTRASTING REPRODUCTIVE SYSTEMS AT THE MICRO-SPATIAL-SCALE OF THE SHORE: PHENOTYPIC AND GENETIC ANALYSES

Billard E. [1,2], Serrão E[2], Neiva J. [1;2], Pearson G.[2], Destombe C.[1] and Valero M.[1], A mosaic of hybrids between species with contrasting reproductive systems at the micro-spatial-scale of the shore: phenotypic and genetic analyses (in prep)

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6.1 Abstract

In the genus *Fucus*, species with contrasting mating systems have been shown to be capable of hybridization, offering a great opportunity to study the evolution of these characters. This is especially true for *F. vesiculosus* and *F. spiralis*. Both species are found in sympatry on European coast from Norway until North Portugal, the hermaphroditic *F. spiralis* living higher on the shore than the dioecious *F. vesiculosus* and hybrids occurring in the contact zone. In the lower part of its distribution, *F. vesiculosus* can also be found in mixture with another dioecious species, phylogenetically less related, *F. serratus*. Our study aims at investigating the relationships between mating system and the speciation/hybridization process at a micro-evolutionary level.

We analyse the pattern of vertical variation in species distribution, allelic frequencies and sexual phenotypes along the micro-spatial-scale of the shore in two locations, Santec (North Brittany) and Viana (North Portugal) in order to investigate the effect of the breeding system on the levels, direction and spatial pattern of introgression.

Our results revealed that dispersal was very limited but sufficient to result in hybridisation when individuals of the different species came into contact, whatever their reproductive systems (either *F. spiralis, F.vesiculosus* or *F. serratus*). However, when the hybridisation process involved partners with different breeding systems, the direction of the crosses was preferentially: sperms of the dioecious species fertilizing the ovules of the hermaphrodite species. Unexpectedly, we discovered the occurrence of two genetically divergent entities differing in their spatial distribution on the shore within *Fucus spiralis*. These two hermaphroditic entities of *F. spiralis* were characterised by high values of F_{IS} suggesting high selfing rates. Finally, the phenological analysis suggested that a temporal discrepancy may occur vertically, limiting gene flow between lower and higher parts of the shore.

All these results were discussed in the context of the difference in selective pressures that occur between the lower and higher parts of the shore. We propose that genetic barriers have arisen because of ecological divergence along the shore and that different mating system has evolved in the context of reinforcement of sympatric speciation.

Keywords: Brown algae, ecological divergence, Fucus, hybridization, mating system, speciation, sexual phenotype

6.2 Introduction

Due to their high diversity of reproductive systems, ranging from hermaphroditism (cosexual individuals) to dioecy, where individuals carry only one type of sexual organ, plants have been extensively studied to understand the evolution of reproductive strategies (see for review Geber et al., 1999). Based on theoretical, empirical and phylogenetic studies, dioecy generally appears to be the derived state from cosexuality in angiosperms. Charlesworth (Charlesworth, 2006) has recently reviewed the importance of breeding system for the evolution of genomes. It has been shown that hermaphroditism via selfing may present a real advantage compared to dioecy by increasing reproductive assurance (Baker, 1955; Jain, 1976); (2) maintaining co-adapted gene complexes (e.g., populations of plants tolerant to heavy metal pollution present higher self-fertility than their relatives, (Antonovics, 1968), (3) transmitting whole genome through both the male and female functions to the next generation (Fisher, 1941). However, selfing leads to a rapid increase of homozygotes in a population, which may have different effects. First, homozygozity may expose to selection recessive deleterious mutations in individuals which may thus suffer from inbreeding depression, but at the end, this exposure of deleterious alleles may induce a purge of this mutational load (Byers & Waller, 1999). On the other hand, a population of homozygotes will have an effective size half of that of an outcrossing diploid population with the same number of individuals. As a consequence, effect of genetic drift may increase, while natural selection's ability to purge deleterious mutations and increase favourable ones may be lowered. In this context, dioecy is thought to have evolved to avoid inbreeding depression in particular in habitats where competition is strong. Moreover, from an ecological standpoint it can be more advantageous to be unisexual when resources are limited (Charnov, 1979). Although many seaweed genera contain hermaphroditic and dioecious species (*e.g. Oedogonium*, *Porphyra Phyllophora*, *Bryopsis*, *Fucus* (Brawley & Johnson, 1992; see also Hawkes, 1990), comparatively few studies have explored the evolution of reproductive strategies in marine algae.

In the intertidal habitat, selective pressures are though to be predominantly of different nature along the shore. They have been suggested (e.g., Vernet & Harper, 1980) to be mostly biotic in the lower zone like interspecific competition or predation, whereas in the upper zone they should be abiotic, species having to resist desiccation or more important temperature and salinity fluctuations. According to Vernet & Harper (1980), abiotic pressure will favour

selfing or asexuality in the upper part of intertidal habitat, whereas in the lower zone, biotic pressure will favour outcrossing.

Hybridization has been described as an invasion of the genome (Mallet, 2005), particularly in plants, where species boundaries are known to be weaker than in animal species. Natural hybrid zones maintained by environment dependent selection may be "ideal laboratories" for studying the role of environment stress in adaptation and speciation (Barton, 2001; Harrison, 1990 for review). Thus, hybrid zones between species with contrasting mating systems offer great opportunities to study evolution of reproductive system under the constant arrival of "mutant" sex determining genes. The maintenance of either or both reproductive strategies depends on equilibrium between interspecific gene flow, which introduces novel variation, and selection against introgression, which maintain species boundaries. While changes in the mating system can influence the degree of reproductive isolation of the parental species, reproductive isolation may also evolve due to divergent selective pressures in different habitats (e.g. Dorken, 2003). Indeed, the role of ecological divergence in sympatric speciation is receiving more acknowledgement and attention. In the marine environment, subtidal and intertidal, ecological divergence has been shown to be involved in the speciation of the annelid *Scoloplos armiger* (Kruse et al., 2004) and of the gastropod genus *Littorina* (Johanneson, 2003).

In the ubiquitous, ecologically-successful brown seaweeds of the genus *Fucus* (Fucales, Phaeophyceae) the character dioecy is though to have evolved from an ancestral hermaphroditism (Billard et al., 2005b;, Billard et al., submitted). Nevertheless, maybe due to their recent radiation, taxa possessing contrasting mating systems are still reported to hybridize in this genus. For example, hybridization has long been reported between hermaphroditic *F. spiralis* and dioecious *F. vesiculosus,* based on morphological evidence (Scott et al., 2000), laboratory crosses, (Kniep, 1925), and more recently *in natura* molecular evidence (Engel et al., 2005; Wallace et al., 2004 but see Wallace et al., 2006, Engel et al., 2006) and between hermaphroditic *F. evanescens* and dioecious *F. serratus* (Coyer et al., in press; Coyer, 2002). Although Engel et al (2005) had based their sampling on a typical-morphology selection of individuals; actually 8% of sampled Fucus were assigned to a cluster of intermediate genotypes. Moreover, hybrids between *F. vesiculosus* and *F. spiralis* have been shown to have a prezygotic fertility as high as their parents (Billard et al., 2005b). This suggests that hybrids are capable of backcrossing and thus that introgression (transfer of a portion of one species genome into another) is possible in one or both species. Despite these possibilities of hybridization, stability of reproductive systems has always been reported with

the exception of *F. ceranoides* (Hamel, 1939) and integrity of genome seems preserved. The contrasted landscape formed by the rocky intertidal habitat allows processes of hybridization and ecological divergence to take place between two species with contrasting reproductive systems at the very restricted scale of hundred meters. It offers thus the opportunity of studying factors settling genetic barriers in non completely isolated species.

A genetically based characterization of species and putative hybrids within sister species in the genus Fucus was conducted by Engel et al (2005) by comparing typical morphological individuals of the species *F. vesiculosus* and *F. spiralis* from two regions (North of Portugal and North of France) sampled in parapatric (discontinuous) or sympatric (continuous) zones. A Bayesian analysis based 5 microsatellite loci revealed that *F. vesiculosus* and *F. spiralis* form distinct genetic entities, consistent with sexual phenotype of individuals: 93% of *F. spiralis* were hermaphroditic and 96% of *F. vesiculosus* were dioecious. Poor genetic diversity and high F_{IS} within *F. spiralis* suggests important reproduction through selfing in this species while in *F. vesiculosus* inbreeding is suggested. Nevertheless, gene flow between these two species are indicated by the occurrence of aberrant sexual phenotype in each cluster, the absence of diagnostic allele and the assignment of 10% of the individuals to intermediate cluster. The fact that significantly more intermediate individuals were found in sympatry than in parapatry and the monomorphy of *F. spiralis* for three loci allowed the authors to reject the alternative hypothesis of ancestral polymorphism to explain this pattern. They concluded that, although introgression exists between species their genome integrity may be due to the divergent breeding systems.

Indeed, in a complementary study (Billard et al, 2005) focused on the sexual phenotypes and resource allocation observed in both species and their hybrids, we showed the importance of selfing in *F. spiralis* and inbreeding in *F. vesiculosus*. Our study was conducted on individuals previously analysed by Engel et al. (2005). A total of 89 mature individuals were analysed: 20 *F. spiralis*, 40 *F. vesiculosus* and 19 hybrids. Sexual phenotypes were observed on six conceptacles per individual with a microscope and resource allocation was estimated with the density of gametes per section of conceptacle. Very little within-individual variability was observed, either qualitative or quantitative. As predicted in the ecological model of mating system evolution (Charnov, 1979) a reallocation of resource towards male function was shown in the dioecious species whereas the hermaphroditic one presented a very low sperm: egg ratio, typical of obligate selfer in flowering plants. Hybrids presented all three

sexual phenotype and showed no fertility decrease. It suggests a simple genetic mechanism of sex determination and the possibility of backcrosses.

These two papers showed the persistence of two distinct species with contrasting reproductive systems despite the possibility of producing fertile hybrids, probably due to their breeding system. At present, more molecular markers, nuclear and cytoplasmic, are available (Perrin et al., 2007). It would thus be interesting to determine, when individuals are sampled at random, what is the impact of these different breeding systems on the extent of hybridization and also on the direction of hybridization, since it can be traced by maternally inherited cytoplasmic markers. Moreover, zonation of species on the shore suggests that environmental factors may exert selective pressure on species, and it thus seems important to take the spatial, vertical distribution into account by mapping and analysing individuals sampled from the lower to the upper part of the Fucus distribution range along the shore.

Finally, although morphological characters like sexual phenotypes have been reported as very stable, they have not been verified individually through time or over a large number of receptacles per individuals. In addition, it is interesting for studying gene flow between populations or species, to couple genetic analysis with an analysis of phenology of these populations.

In order to approach these questions, we studied populations of *F. spiralis* and *F. vesiculosus* in two locations along their distribution range: one in Brittany, France and one in North Portugal, which is the southern limit of sympatry between the two species, since it is the southernmost region where the dioecious *F. vesiculosus* is found in open coast. The specific aims of this study were thus to analyse: 1) the number of genetic clusters in random samples without taking species identification into account, 2) intensity and orientation of hybridization, 3) genetic structure at a fine spatial scale along the shore, 4) stability of sexual phenotype and phenology within species and individuals, 5) relationships between breeding system and sexual phenotypes.

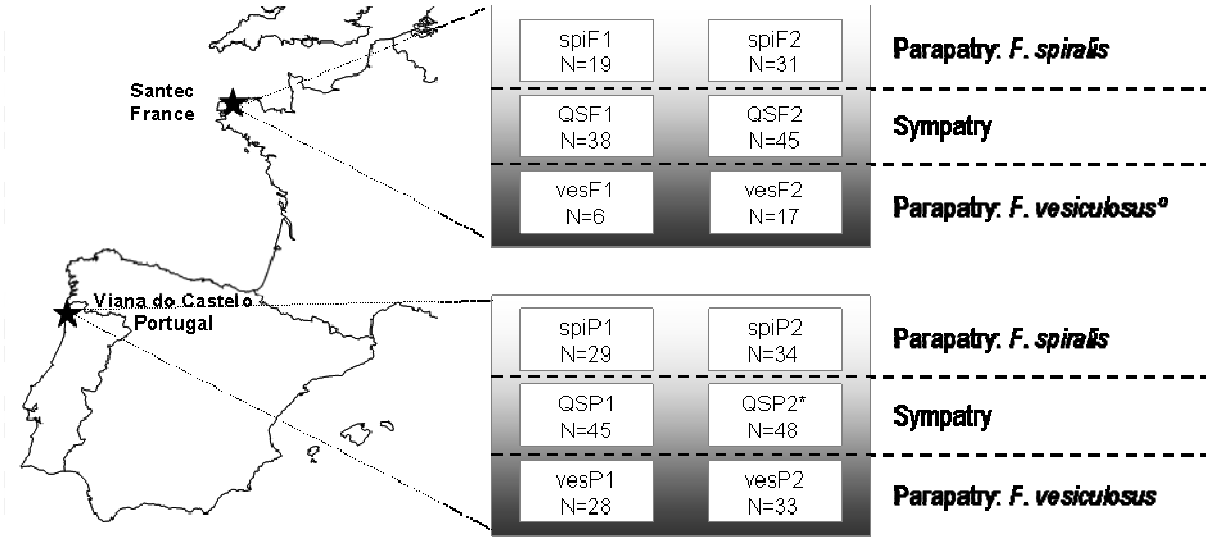
6.3 Materials and Methods

6.3.1 Sampling design

Sampling of individuals was conducted in Santec (Brittany, France) and in Viana do Castelo (North Porugal) (Fig 1). In each region, two kinds of sampling were performed: quadrats and transects.

Quadrat sampling: individuals of *F. vesiculosus* and *F. spiralis* (hereafter called ves and spi) were sampled randomly within quadrats (Fig.1 A). Two situations were considered: parapatric situation, where one species occurs without the other one and sympatric situation, when both species are found in contact. In each region, two parapatric quadrats were sampled between 2005 and 2006 for each species, spiP1, spiP2 (spi from Portugal), spiF1, spiF2 (spi from France), vesP1, ves P2 (ves from Portugal) vesF1 and vesF2 (ves from France) as well as two sympatric quadrats: QSP1 (Quadrat Sympatry Portugal 2005), QSP2 (Quadrat Sympatry Portugal 2006), QSF1 (Quadrat Sympatry France 2005) and QSF2 (Quadrat Sympatry France 2006). All parapatric quadrats from Portugal were sampled in 2006 while quadrats from Fransect were sampled in 2005 and 2006 for each species. In the field, *a priori* taxonomic identification of individuals was based on the presence/ absence of vesicles and confirmed in the lab by checking their reproductive system under a microscope. In Portugal, after checking sexual phenotypes, QSP2 was revealed as composed almost exclusively of unisexual individuals with or without vesicles. Consequently, it was not considered as sympatric in the analyses. In France, *F. vesiculosus* could not be found alone and therefore what is called parapatric quadrats in Santec is actually sympatric quadrats of *F. vesiculosus* and *F. serratus* (ser). The later species is much more easily distinguishable from the other species on a morphological basis (shape of the thallus).

A: quadrats



B: transects

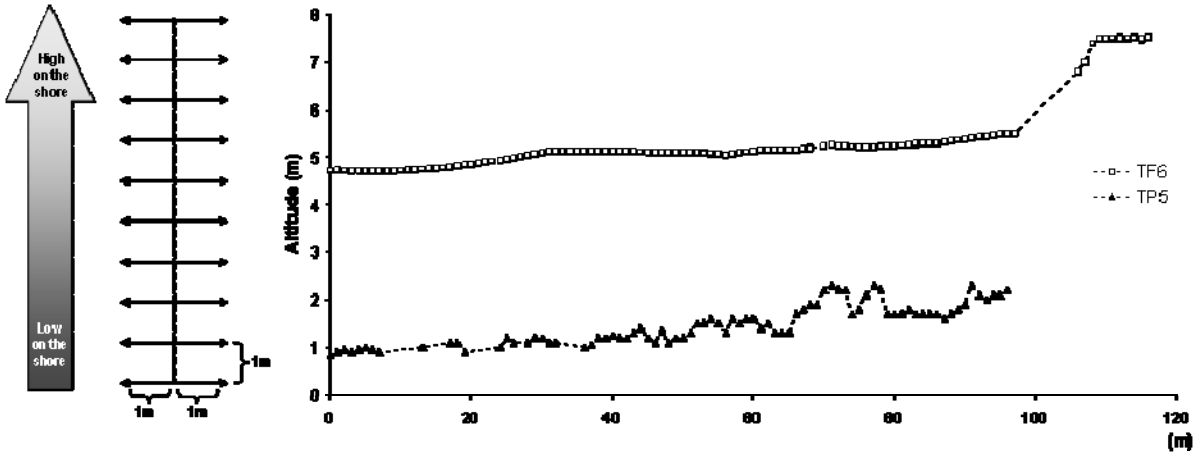


Figure 1: Two regions were sampled: Viana do Castelo in Portugal and Santec in France. In each region, two kinds of sampling were conducted:
A represents quadrats where *Fucus* individuals were sampled randomly regardless of species: parapatric quadrats (in areas where *F. vesiculosus* and *F. spiralis* are non contiguous) spiF1, spiF2 (*F. spiralis* France 1 & 2), spiP1 and spiP2 (*F. spiralis* Portugal 1 & 2), and sympatric quadrats (in areas where *F. vesiculosus* and *F. spiralis* occur jointly) QSF1, QSF2 (quadrat sympatry France 1 & 2), QSP1 and QSP2 (quadrat sympatry Portugal 1 & 2). Quadrats vesF1 and vesF2 were made among the populations of *F. vesiculosus* mixed with the populations of *F. serratus,* as there were no "pure" *F. vesiculosus areas at the site*. QSP2* was conducted in a sympatric area, but the random sample turned out to be actually composed almost exclusively by *F. vesiculosus*.
B represents sampling along transects: two transects were sampled in each site, one in 2005 and one in 2006. Two individuals were taken randomly each meter from low to high shore. Profiles of transects TP5 (transect Portugal 2005) and TF6 (transect France 2006) are shown. Tidal amplitude in Santec (TF6) is 10m with mid-tide at 5m while in Viana (TP5) it is 3m with mid-tide around 2m

Transect sampling: Individuals were sampled randomly along a vertical transect (Fig. 1B) from the lower to the higher limits of the intertidal zone. Two individuals were taken at every meter along the transect, one at 1 m to the left and one at 1 m to the right right. These transects were sampled in 2005 and 2006 in both regions (Transect France 2005 (TF5) L = 120m, n=92/ 217, Transect France 2006 (TF6) L = 87m, n = 150, Transect Portugal 2005 (TP5) L = 83m, n=160, Transect Portugal 2006 (TP6) L = 71m, n = 105). Vegetative tips (2 – 3 for each individual) were stored in silica gel for future molecular analyses and at least two receptacles (fertile zones) per individual were taken to check the sexual phenotype.

6.3.2 DNA extraction, PCR reaction and genotyping

DNA for genotyping was extracted from ca. 4 mg of dried tissue using the Nucleospin® Multi-96 plant kit (Macherey-Nagel) according to the manufacturer's protocol and diluted 1:250. Polymerase chain reactions (PCR) and electrophoresis of PCR products for loci L20, L38, L94, L58, L78 and fsp1, fsp2 were performed as described in Engel et al. (2003) and Perrin et al (2007) respectively.

6.3.3 Chloroplast DNA genotyping

Regions of interest were selected on the basis of the completely sequenced chloroplast genome of *F. vesiculosus* (Pearson, unp). We identified one intergenic region presenting two series of single nucleotide repeats, since chloroplast-encoded simple sequence repeats are known for their polymorphism in higher plants (e.g. pines, (Vendramin, 1996 #1572)). These single nucleotide repeats were separated by 40 base pairs, it was thus impossible to define primers for each region. However, it was possible to define primers containing both regions using Primer3 software (Rozen, 2000 #1154) and then to use the restriction enzyme Ase1 to cut PCR products. For the PCR amplification both forward and reverse primers were fluorescently labelled. PCRs were performed in 20µL containing 0.1µg/µL bovine serum albumin, 75mM Tris-HCl, 20mM $(NH_4)_2SO_4$, 0.01% Tween®20, 2.5mM $MgCl_2$, 0.20µM of each forward and reverse primer, 0.05µM labelled primers, 200µM of each dNTP, 0.5U Thermoprime Plus *Taq* polymerase (ABgene) and 5µL of diluted DNA. PCRs were run on a PTC200 thermocycler (MJ Research). After an initial denaturation step (95°C, 5min), 'touchdown' PCR was carried out for 5 cycles of 30s at 95°C, 30s at 48°C, reduced by 1°C

per cycle for 6 cycles, and 30s at 72°C, followed by 30 cycles of 95°C for 30s, 42°C for 30s and 72°C for 30s and a final 7 min elongation at 72°C. After restriction, fragments were analysed on an automated DNA sequencer (Li-COR 4200^{TM}). The second region used was the rubisco spacer. Indeed, we observed that *F. spiralis* and *F. vesiculosus* presented one mutation difference (genbank). This mutation can easily be detected for the resulting sequence in *F. spiralis* since it is recognised by the enzyme Ssp1, whereas the sequence of *F. vesiculosus* is not. Primers and PCR conditions used are described in Billard et al (in prep). Electrophoresis was performed on agarose gel (2%).

6.3.4 Genetic analyses

Existence of null alleles was tested with MICRO-CHECKER (Van Oosterhout et al, 2003, 2006). Genetic analyses of species were performed using Genetix software Belkhir and Genepop (version 1.2 Raymond & Rousset, 1995). First, parapatric populations were considered to compare allelic frequencies and genetic distances between species. We calculated the mean number of allele per locus (A) and average non biased expected heterozygosity (H_E) for each quadrat separately. F_{ST} were calculated between all pairs of populations. Four levels of comparison were thus considered; comparison of populations within species and within regions, populations within species between regions, populations between species within regions and populations between species between regions. Correspondence analysis (CA) based on the data obtained from the transects was performed using the AFC procedure implemented in the GENETIX software. All individuals with missing data at one or more loci were excluded. To analyse the mating system fixation indices, F_{IS} (Weir & Cockerham), were calculated over all loci and for each locus within each population. Heterozygote deficiencies and excess were tested using 10000 permutations of alleles among individuals within each population

Clustering assignments were firstly performed on parapatric and sympatric quadrats. Assignment of individuals to species and detection of hybrids was performed with a model-based genetic admixture analysis implemented in the Structure software (Pritchard et al. 2000). This software uses a Bayesian method to identify clusters of genetically similar individuals based on their multilocus genotypes by creating groups within which linkage disequilibrium is minimized. It assigns individuals to the different clusters it creates and detects admixed individuals resulting from recent hybridization and/or introgression of these

clusters. Although inbreeding may induce linkage disequilibrium among loci and Hardy-Weinberg disequilibrium which may not be suitable for assignment tests, sufficient differences among taxa provide robustness (see Engel et al, 2003). Analyses were run assuming different numbers of parental populations from K=2 to K=5 with 8 iterations for each assumed K. A burn-in of 100000 repetitions and a run length of 500000 were used. To avoid potentially confounding geographic structure, analyses of quadrats and transect were carried out separately for each region. Individuals were then assigned to each cluster according to q_t^i , the proportion of individual genome assigned to each taxon. An individual was considered as hybrid when q_2^i (the proportion of genome assigned to the second taxon) $\geq$ 0.1. Due to the presence of *F. serratus* in the French transects, percentages of hybrids between *Fucus vesiculosus* and *Fucus spiralis* could have been underestimated if they were calculated over the total number of sampled individual. Thus we eliminated *F. serratus* individuals for the calculation of percentage.

6.3.5 Spatial auto-correlation

Spatial auto-correlation was used to examine the organisation of the genetic variation at the within-population level. Pairwise genetic correlation between individuals is expected to decrease in a more or less linear fashion under an isolation-by-distance process in a two dimensional spaces (Hardy and Vekemans 1999, Rousset 2000). To estimate pairwise genetic correlation between individuals, we used a multiallelic, multilocus relationship coefficient, Moran's I statistic, which has the advantage of being selfing-rate-insensitive. Multi and single locus pairwise Moran's I relationship coefficients were calculated for each cluster, separately for each transect, and regressed on pairwise separation distance, with SpaGeDi (Hardy and Vekemans 2002). Only the numbers of distance class were predefined, lengths were calculated by spaGeDi in order to homogenise the number of pair comparisons within each class. Slopes were noted for each polymorphic locus as well as for multilocus and null hypothesis of random spatial distribution was rejected when $P<0.05$.

6.3.6 Phenotypic analyses

For all individuals, the first criterion observed was the presence or not of vesicles (table 1), this observation was performed directly in the field. Individuals from 2006 transects

were measured in length and perimeter. Moreover, in Santec these measures were carried out every two months.

6.3.7 Sexual phenotype analysis.

Sexual phenotype of all individuals was verified by observation under a microscope of sections of receptacles. To check for among-receptacle variation within individuals, all receptacles were observed on 187 individuals in Portugal and 57 in France (table 1) and the potential temporal variation was assessed by observing two receptacles per individual each month in France during a nine-month-survey in 2006, to detect any gender and maturity state change. Sex ratio were calculated and compared to a binomial probality (p=q=0.5). Statistical analyses were performed in Minitab (version 13.2 MiniTab Inc. 1994, State College USA).

Table 1: Summary of the analyses performed on individual phenotypes (vesicles and receptacles).

Study sites		France			Portugal			Total
	Years	Transects	Quadrats	Total	Transects	Quadrats	Total	
Morphological analyses° (N ind)	2005	217	80	397	160	50	210	607
	2006	199	76	275	105	137 + 50	292	567
	Total	416	156	**672**	265	237	**502**	**1174**
Sexual phenotype° (N rec /N ind)	2005	250 / 125	66 / 33	316	296 / 160	74 / 37	370	686
	2006	* 656 / 199	796 / 57	1452		3006 / 187	3006	4458
	Total	906 / 324	862 / 90	**1768**	296 / 160	3080 / 224	**3376**	**5144**

° Morphological (presence and absence of vesicles) and sexual phenotypes (receptacles) observations were conducted on the same individuals, when mature.
*During the 9 month survey, 2 receptacles were observed per individual each month

6.4 Results

6.4.1 Genetic analyses

✓ Quadrats

Individual genotypes were determined at seven microsatellite loci on 200 individuals. All loci presented a substantial polymorphism with a number of alleles ranging from 7 to 13. This polymorphism was different according to the taxon considered with 5-7 alleles in *F. vesiculosus* populations and 1-2 in *F. spiralis* populations (table 2A). The number of *F. vesiculosus*-private alleles range from 14 to 39 with 10 to 25 at a frequency >0.05 while the maximum number of *F. spiralis* private alleles was 3, observed in the French site. *F.*

vesiculosus populations of France presented twice as many private alleles as populations of Portugal.

Table 2: A, Summary of genetic diversity among parapatric populations. N, number of individuals; H_E total expected heterozygosity (SE); H_O total observed heterozygosity (SE); P(0.95), proportion of polymorphic loci (where most common allele does not exceed 0.95) A, mean number of alleles per locus; SSA, species-specific (private) Allele; SSA (0.05); number of species-specific alleles at a frequency >0.05. i= irrelevant value since there were only 6 individuals, all alleles occurred at a frequency >0.05. vesP1, ves P2: *F.vesiculosus* from Portugal, vesF1 and vesF2: *F.vesiculosus* from France, spiP1, spiP2: *F. spiralis* from Portugal, spiF1, spiF2: *F. spiralis* from France. B, Locus by population F_{IS} (W&C) estimates

A

POP	*N*	H_E	H_O	*P(0.95)*	*A*	*SSA*	*SSA (>0,05)*
ves P1	29	0.588 (0.103)	0.535 (0.162)	1.000	4.9	19.0	10.0
ves P2	34	0.600 (0.065)	0.514 (0.105)	1.000	4.0	14.0	11.0
ves F1	6	0.593 (0.147)	0.520 (0.190)	1.000	4.8	18.0	i
ves F2	22	0.737 (0.090)	0.652 (0.148)	1.000	7.1	39.0	25.0
spi F1	19	0.134 (0.193)	0.037 (0.099)	0.143	1.3	1.0	0.0
spi F2	31	0.064 (0.206)	0.039 (0.088)	0.429	1.9	3.0	3.0
spi P1	27	0.071 (0.205)	0.038 (0.036)	0.571	2.1	1.0	1.0
spi P2	33	0.106 (0.108)	0.052 (0.067)	0.143	1.6	1.0	0.0

B

	F_{IS}							
	over all loci	**L20**	**L38**	**L58**	**L78**	**L94**	**fsp2**	**fsp1**
vesP1	0.1558***	0.224*	0.132	-0.04	-0.028	-0.043	0.562***	-0.021***
vesP2	0.1838***	0.402***	0.044	0.164	0.066	0.027	0.437***	-0.129***
vesF2	0.1637**	0.047	0.195*	-0.017	0.167*	0.052	0.283*	0.236*
spiP1	0.7324***	0.662*	------	0	0.662***	0.816***	------	0.658***
spiP2	0.4000***	------	------	------	0.402*	0	1***	-0.016
spiF1	0.4915***	------	------	------	------	------	0.492*	------
spiF2	0.5203***	-0.024	------	------	0.659***	------	0.59***	------

Fixation indices F_{IS} revealed deficiency of heterozygotes for three loci in *F. vesiculosus* populations: L20, fsp1 and fsp2, (Fig.2B) null alleles were suspected to occur for these loci when analysed with Microchecker. Within *F. spiralis*, F_{IS} were calculated on a maximum of 4 loci per population, the other loci being fixed. They were highly variable according to locus considered. Details of allele frequencies and heterozygosity per locus are given in table 3. Within *F. spiralis* four loci - L20, L78, L94 and fsp1- presented a switch in the most frequent allele. Moreover a private allele (120) is observed at L20, in the Portuguese population spiP1. In addition, the distribution of allele frequencies differs significantly among

the regions, as shown by the F_{ST} calculated between all pairs of populations (fig. 2). The compared pairs were:

Within species, within regions		Within species, between regions		Between species, within regions	Between species, between regions
ves	spi	ves	spi		
vesP1-vesP2	spiP1-spiP2	vesP1 - vesF	spiP1 - spiF1	spiP1 - vesP1	spiP1 - vesF
	spiF1-spiF2	vesP1- vesF	spiP1 - spiF	spiP1 - vesP2	spiP2 - vesF,
			spiP2 - spiF1	spiP2 - vesP1	spiF1 - vesP1
			spiP2 - spiF	spiP2 - vesP2	spiF1 - vesP2
				spiF1 - vesF1	spiF2 - vesP1
				spiF2 - vesF	spiF2 - vesP2

Because the sample size of vesF1 was very small, quadrat was pooled with vesF2 after checking that F_{ST} between the two populations of was not significant. All F_{ST} values were highly significant, except for the pair spiF1- spiF2. Within *F. vesiculosus*, F_{ST} were ten times higher between than within regions for all loci except L78 and fsp2 . On the other hand, the pattern was different within *F. spiralis* and highly dependent on the locus considered. Most of the loci showed fixed alleles within quadrats leading to very contrasted values of F_{ST} (closed to 0 when the same allele was shared by pairs or to 1 when different alleles were fixes). Two loci, L38 and L58 were monomorphic, locus fsp2 showed no within and a high between region differentiation while and L20, L78, fsp1 and L94 presented high F_{ST} between the spiP1 and all other quadrats of *F. spiralis*, suggesting the occurrence of two different genetic entities within *F. spiralis*.

Table 3: allele frequency in parapatric quadrats of *F. vesiculosus* (ves) and *F. spiralis* (spi) in Portugal (P) and France (F). Private alleles are indicated in bold. Expected (H exp.) and observed (H obs.) heterozygosity is indicated for each population and each locus.

L20	ves P1	ves P2	ves F1	ves F2	spi P1	spi P2	spi F1	spi F2
N	28	34	6	17	27	33	19	30
120	0	0	0	0	**0.94**	0	0	0
132	0.05	0.04	0	0	0	0	0	0.02
138	0	0.01	**0.08**	**0.21**	0	0	0	0
141	**0.13**	**0.28**	**0.17**	**0.29**	0	0	0	0
144	0	0	**0.08**	**0.21**	0	0	0	0
147	0.54	0.49	0	0	0.04	1.00	1.00	0.95
150	0.09	0.07	0.08	0.09	0.02	0	0	0
153	0	0	0.42	0.09	0	0	0	0.03
156	**0.04**	0	0	**0.09**	0	0	0	0
159	**0.05**	**0.03**	**0.17**	0	0	0	0	0
162	0	0	0	**0.03**	0	0	0	0
165	0	**0.01**	0	0	0	0	0	0
168	**0.11**	**0.06**	0	0	0	0	0	0
H exp.	0.6831	0.6844	0.818	0.8289	0.1045	0	0	0.0947
H obs.	0.5	0.4118	0.6	0.8235	0.0357	0	0	0.0968

L38	ves P1	ves P2	ves F1	ves F2	spi P1	spi P2	spi F1	spi F2
N	32	34	6	17	27	33	19	30
163	0	0	0	**0.03**	0	0	0	0
166	0	0	0	**0.03**	0	0	0	0
169	0.66	0.54	0.33	0.26	1.00	1.00	1.00	1.00
172	0	0	0	**0.09**	0	0	0	0
181	**0.06**	0	0	0	0	0	0	0
190	**0.25**	**0.34**	**0.25**	**0.32**	0	0	0	0
193	0	0	**0.08**	**0.06**	0	0	0	0
196	0	0	0	**0.12**	0	0	0	0
199	0	0	**0.17**	0	0	0	0	0
205	0	0	0	**0.06**	0	0	0	0
208	**0.03**	**0.12**	0	0	0	0	0	0
223	0	0	0	**0.03**	0	0	0	0
H exp.	0.5099	0.5843	0.833	0.8182	0	0	0	0
H obs.	0.4063	0.5588	1	0.5882	0	0	0	0

L58	ves P1	ves P2	ves F1	ves F2	spi P1	spi P2	spi F1	spi F2
N	30	34	6	17	27	33	19	30
105	0	0	0	**0.03**	0	0	0	0
107	0.22	0.34	0.25	0.26	0.98	1.00	1.00	1.00
109	0.72	0.53	0.33	0.18	0.02	0	0	0
111	0	0	**0.42**	**0.29**	0	0	0	0
113	0	0	0	**0.03**	0	0	0	0
115	**0.07**	**0.13**	0	**0.03**	0	0	0	0
119	0	0	0	**0.03**	0	0	0	0
121	0	0	0	**0.06**	0	0	0	0
135	0	0	0	**0.03**	0	0	0	0
155	0	0	0	**0.03**	0	0	0	0
183	0	0	0	**0.03**	0	0	0	0
H exp.	0.4424	0.5966	0.7121	0.8271	0.036	0	0	0
H obs.	0.4667	0.5	0.8333	0.7647	0.036	0	0	0

L78	ves P1	ves P2	ves F1	ves F2	spi P1	spi P2	spi F1	spi F2
N	31	34	5	16	27	33	19	30
122	0	0	0	0.03	0.04	0.82	1.00	0.95
131	0	0	0	0	0	0	0	**0.05**
134	0.05	0	0	0.03	0.02	0	0	0
137	0.03	0.01	0.08	0.03	0.94	0.18	0	0
149	0	0	0	**0.06**	0	0	0	0
152	**0.56**	**0.40**	**0.42**	**0.34**	0	0	0	0
155	**0.35**	**0.59**	**0.33**	**0.28**	0	0	0	0
158	0	0	**0.17**	0	0	0	0	0
161	0	0	0	**0.16**	0	0	0	0
179	0	0	0	**0.06**	0	0	0	0
H exp.	0.5611	0.5035	0.7424	0.7923	0.1083	0.3021	0	0.0936
H obs.	0.5806	0.4706	0.8333	0.5625	0.037	0.1818	0	0.0323

L94	ves P1	ves P2	ves F1	ves F2	spi P1	spi P2	spi F1	spi F2
N	32	34	6	17	28	33	19	30
136	0.03	0	0	0	0.55	0	0	0
139	0	0	0	0	**0.13**	**0.02**	0	0
145	0.25	0.25	0	0	0.32	0.98	1.00	1.00
151	0	0	**0.08**	**0.06**	0	0	0	0
154	**0.42**	**0.40**	**0.50**	**0.59**	0	0	0	0
160	**0.30**	**0.35**	**0.25**	**0.21**	0	0	0	0
163	0	0	0	**0.03**	0	0	0	0
166	0	0	**0.08**	**0.03**	0	0	0	0
169	0	0	0.0833	**0.09**	0	0	0	0
H exp.	0.6811	0.6651	0.7273	0.6168	0.5851	0.0303	0	0
H obs.	0.6875	0.6471	0.8333	0.5294	0.1071	0.0303	0	0

fsp2	ves P1	ves P2	ves F1	ves F2	spi P1	spi P2	spi F1	spi F2
N	30	32	5	17	27	33	19	30
152	**0.02**	**0.02**	0	0	0	0	0	0
156	0.55	0.45	0.40	0.44	1.00	0.97	0.37	0.23
160	**0.05**	**0.23**	**0.10**	**0.09**	0	0	0	0
164	0.38	0.30	0.40	0.38	0	0.03	0	0
192	0	0	0.10	0.09	0	0	0.61	0.62
194	0	0	0	0	0	0	0	**0.07**
198	0	0	0	0	0	0	**0.03**	**0.08**
H exp.	0.5571	0.6617	0.7333	0.6631	0	0.0597	0.5107	0.5633
H obs.	0.2667	0.375	0.6	0.4706	0	0	0.2632	0.2333

fsp1	ves P1	ves P2	ves F1	ves F2	spi P1	spi P2	spi F1	spi F2
N	30	33	6	17	27	33	19	30
140	0.17	0.18	0.33	0.26	0.06	0.97	1.00	1.00
142	0.13	0.05	0	0.15	0.94	0.03	0	0
144	**0.48**	**0.62**	0	**0.06**	0	0	0	0
146	**0.03**	0	**0.08**	**0.12**	0	0	0	0
148	**0.02**	0	0.17	**0.24**	0	0	0	0
150	**0.10**	**0.15**	**0.42**	**0.15**	0	0	0	0
154	0	0	0	**0.03**	0	0	0	0
160	**0.02**	0	0	0	0	0	0	0
162	**0.05**	0	0	0	0	0	0	0
H exp.	0.7186	0.5646	0.7424	0.8378	0.1069	0.0597	0	0
H obs.	0.7333	0.6364	0.3333	0.8235	0.037	0.0606	0	0

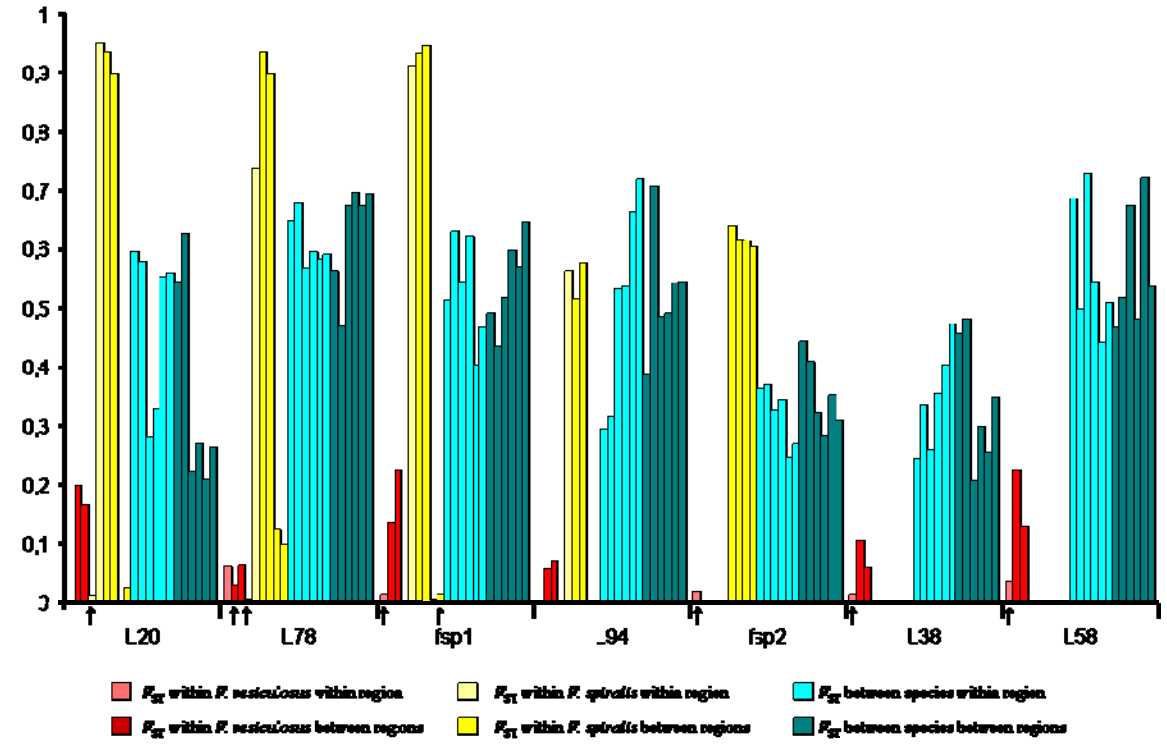


Figure 2: Pairwise F_{ST} within and between species and within and between regions. Non null and non significant F_{ST} are indicated by black arrows. Within each comparison type, compared pairs were, from left to right:

vesP1 -vesP2
vesP1 - vesF, vesP1- vesF
spiF1 - spiF2, spiP1 - spiP2
spiP1 - spiF1, spiP1 - spiF, spiP2 - spiF1, spiP2 - spiF
spiP1 - vesP1, spiP1 - vesP2, spiP2 - vesP1, spiP2 - vesP2, spiF1 - vesF1, spiF2 - vesF
spiP1 - vesF, spiP2 - vesF, spiF1 - vesP1, spiF1 - vesP2, spiF2 - vesP1, spiF2- vesP2

Definition of genetic clusters

The cluster assignment was implemented with the software STRUCTURE (Pritchard et al 2000). A model based genetic admixture was used to assign individuals to clusters and to detect putative hybrids. Due to the high genetic differentiation previously detected between quadrats of *F. spiralis*, we considered two different cases: either two or three parental species to run the analysis (K=2 or K=3) in Portugal and three or four parental species in France, taking into account the presence of *F. serratus*. In both regions, the posterior probability (ln Pr(X/K) was greater when assuming two different cluster within this taxon, one corresponding to spiP1 and the other to spiP2. No admixture between ves and spi was detected for parapatric quadrats (Fig.3) suggesting very low hybridization in parapatric situation. In the sympatric quadrats *F. spiralis* corresponding to spiP1 was found in mixture with *F. vesiculosus* even in the French region where no spiP1 was observed in the parapatric

quadrat (Fig.3), Considering the locations on the shore, the two groups of *F. spiralis* will now be referred as "*F. spiralis* high"=spiH (spiP2, spiF1 and spiF2) and "*F. spiralis* low"=spiL (spiP1 and the sympatric populations).

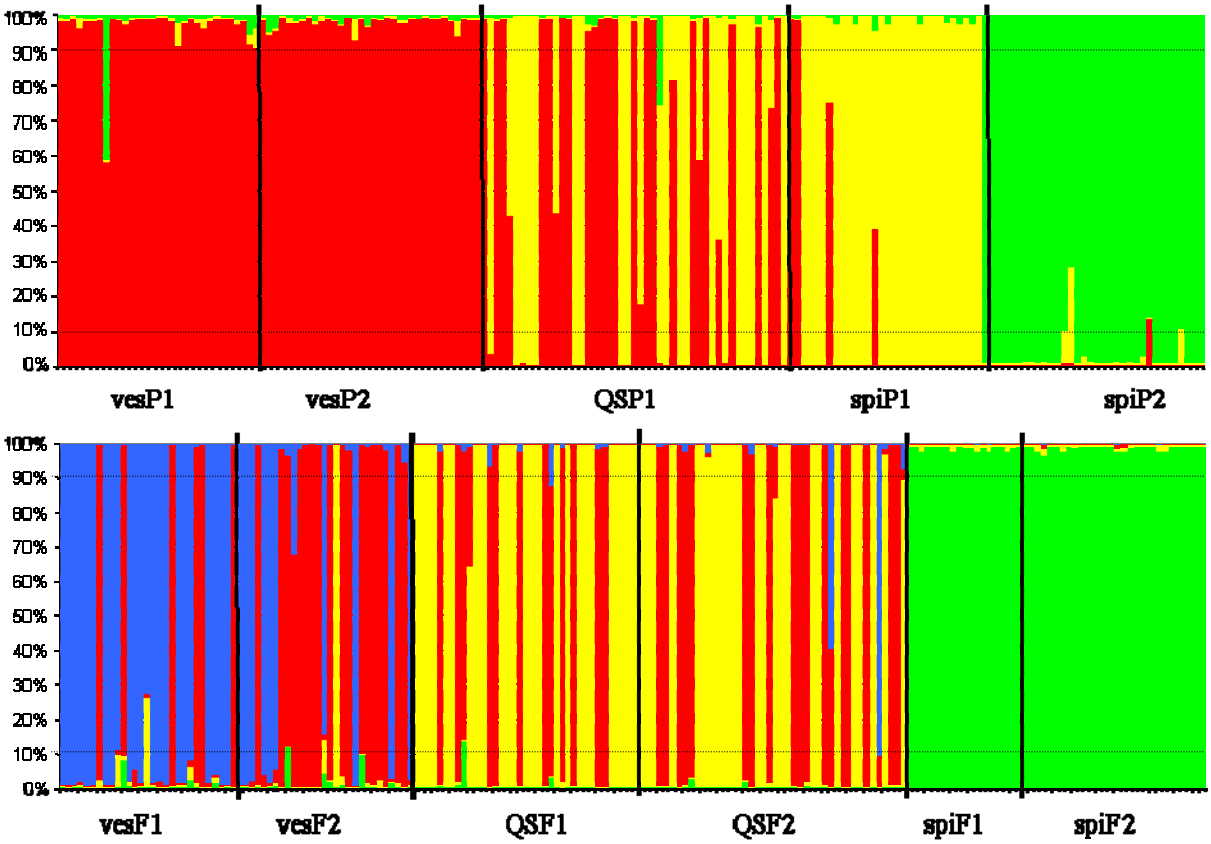


Figure 3: Bayesian analysis with structure software for the sampled quadrats. Each vertical bar represents a different individual. Each colour represent q_t^i , the proportion of individual genome assigned to each taxon; red, *F. vesiculosus*, yellow; *F. spiralis l*; green, *F. spiralis h* and blue, *F. serratus*. QSP1 quadrat sympatry Portugal 2005; QSF1, quadrat sympatry France 2005; QSF2, quadrat sympatry France 2006. Horizontal lines represent the limit of 0.1 (minimum proportion of the 2^d genome for an individual to be considered as hybrid)

In both regions the proportion of hybrids was higher in the sympatric quadrats than in parapatric situation (Fig. 3 and table 4), although it is significant in Portugal (Fisher exact test $P<0.05$) and not in France ($P>0.08$). The level of hybridization tends to be higher in Portugal, the southern limit of sympatry between *F. vesiculosus* and *F. spiralis* than in France ($P=0.03$). The number of hybrids is also greater between *F. vesiculosus* and spiL, than between ves and spiH but in all these samples it was much unusual to find spiH in sympatry with *F. vesiculosus*. It is also noteworthy that hybrids between *F. serratus* and *F. vesiculosus* as well as hybrids between *F. serratus* and *F. spiralis* were found in France. In Portugal no

individuals of *F. serratus* were present near the areas sampled. The spatial distribution of hybrids and of the three taxa was then investigated with the analysis of the transects.

Table 4: Proportions of hybrids between *F. vesiculosus* and *F. spiralis* found in parapatric and sympatric quadrats in Portugal and France. An individual was considered as hybrid when q_2^1 (the proportion of genome assigned to the second taxon) ≥ 0.1 The total number of hybrids is given followed by the number of hybrids between *F. vesiculosus* and each taxon of *F. spiralis* (hybrid ves-spiL/hybrid ves-spiH). The percentages given are the percentages of hybrids ves-spiL and of hybrids ves-spiH.

Sites	Parapatry		Sympatry	
Portugal	124 individuals; 4 hybrids (2/2)		46 individuals; 7 hybrids (7/0)	
% hybrids	1.6 %	0.016%	15%	0%
France	73 individuals; 0 hybrid		83 individuals; 3 hybrids (2/1)	
% hybrids	0.%	0%	2.4%	1.2%

- ✓ Transects

A total of 680 individuals were genotyped at 7 microsatellite loci. The four transects were analysed separately with STRUCTURE (Fig. 4) for the transects in Portugal sampled in 2005 and 2006 (TP5 and TP6), 3 parental species were assumed (fig 4A and 4B) while four (Fig. 4C and 4D) were assumed for the French transects 2005 and 2006 (TF5 and TF6). *F. spiralis* high was found in all transects except in the transect realised in France in 2005. The first part of the French 2005 transect was dominated by *F. serratus*. Due to problems in amplification (possible null allleles) of this later species using the two microsatellite loci specifically developed for spiralis (fsp1 and fsp2) a first analysis on the whole transect was perfomed using five loci only, then the higher part of the transect composed mainly of *F. vesiculosus* (ves) and *F. spiralis* (spi) was reanalysed with 7 loci after removal of the scarce *F. serratus* individuals to check for any difference in the assignment. Individuals considered as hybrids between *F. vesiculosus* and *F. serratus* were assigned to *F. vesiculosus* in the second analysis (with K=2 since there was no spiH). Thus, the two analyses were congruent. Proportion of each taxa found in the different transects is given in table 5. The proportion of hybrids ves* spiL was similar (5.5 to 5.7%) for three transects (TP6, TF5 and TF6) but was more than twice as important in TP5 (12.7%). As well, hybrids ves*spiH were more numerous in this transect. Hybrids spiL*spiH were only observed in TP6.

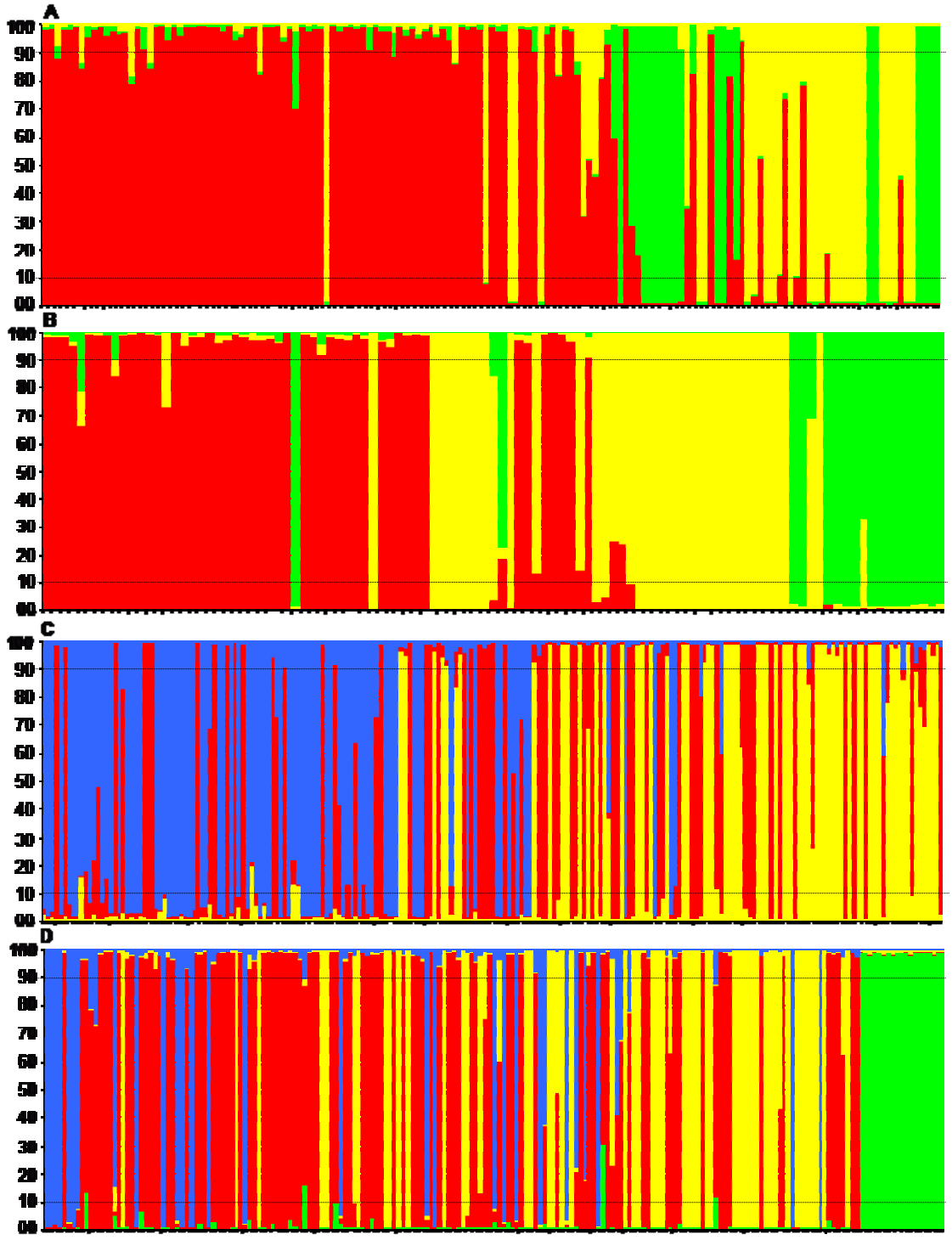


Figure 4: Bayesian analysis with structure software. For the transects sampled in Viana do Castelo, Portugal A: during 2005 (TP5), B: during 2006 (TP6) and in Santec, France C: during 2005 (TF5) D: and 2006 (TF6). Each vertical bar represents a different individual. Each colour represent q_t^i , the proportion of individual genome assigned to each taxon; red, *F. vesiculosus*, yellow; *F. spiralis l*; green, *F. spiralis h* and blue, *F. serratus*. Horizontal lines represent the limit of 0.1 (minimum proportion of the 2^d genome for an individual to be considered as hybrid)

Table 5: Numbers and proportions of individuals assigned to each taxon and to hybrids in the transects sampled in Portugal and France. An individual was considered as hybrid when q_2^i (the proportion of genome assigned to the second taxon) ≥ 0.1.

	Transect Portugal 2005		Transect Portugal 2006		Transect France 2005		Transect France 2006	
F. spiralis high	16	(10.1%)	16	(15.1%)	0		19	(9.5%)
F. spiralis low	27	(17.1%)	33	(31.1%)	58	(26.7%)	48	(24.1%)
F. vesiculosus	87	(55.1%)	47	(44.3%°	58	(26.7%)	84	(42.2%)
F. serratus					68	(31.3%)	25	(12.6%)
spiH x spiL	0		3	(2.8%)	0		0	
spiH x ves	8	(5.1%)	3	(2.8%)	0		4	(2.4%)
spiL x ves	20	(12.7%)	6	(5.6%)	7	(5.7%)	9	(5.5%)
spiL x ser					7	(5.0%)	1	(0.9%)
ves x ser					19	(9.9%)	9	(5.0%)

i. Vertical distribution of clusters

The different taxa were much more mixed in France than in Portugal (Fig.4). Indeed, in the French site, *F. vesiculosus* area is completely mixed with *F. serratus* in the lower part and with spiL in the upper part of its distribution on the shore (Fig.5). In both sites, *F. spiralis* high is found clearly much higher on the shore than the other taxa, there is no overlapping in the distribution of the two types of *F. spiralis*. In Viana do Castelo Pavia et al., , the zonation of each cluster appeared well defined, with hybrids located between each parental species (Fig. 4C, 4D and Fig. 5). The situation in Santec was not so clear: since *F. serratus* and *F. vesiculosus* were not clearly separated hybrids between *F. serratus* and *F. vesiculosus* were not significantly located below the *F. vesiculosus* area. On the other hand, hybrids between *F. vesiculosus* and *F. spiralis* Low were found significantly above the *F. vesiculosus* area but not significantly below *F. spiralis* (General Linear Model, P≤0.001 & P=0.168 respectively). Hybrids ves*spiL seemed thus to be found mainly within the *F. spiralis* populations.

ii. Orientation of inter-specific crosses

The analyses of the Rubisco-spacer chloroplastic marker, allowing to distinguish between *F. spiralis* and *F. vesiculosus* (Billard et al submitted) (table 6 A) showed that the totality of individuals assigned to *F. spiralis* (low and high) based on their nuclear genotype contained a *spiralis* haplotype (Hsp). In *F. serratus* cluster, 96% of individuals had a *serratus* haplotype (Hse) and in *F. vesiculosus* cluster, only ¾ of individuals had a *vesiculosus* haplotype (Hve) while ¼ had a *spiralis* haplotype Hsp. The ves*spiL hybrids possessed either

a *spiralis* or a *vesiculosus* haplotype (86% and 14% respectively), whereas the ser*ves hybrids possessed the three kind of haplotype (67% Hve, 22% Hsp and 11% Hse). The analysis of the other chloroplastic marker psbD-rpl19 revealed 5 different haplotypes, namely H1 to H5 (table 6B), with H1 being highly dominant in all clusters. A first classification of haplotypes was done according to the Rubisco spacer, and then the different haplotypes psbD-rpl19 were reported within each Rubisco haplotype. The combination H1-Hsp haplotype was present in all clusters. Moreover, the other psbD-rpl19 haplotypes were all restricted to Hsp haplotype except H3 which was present in only one *F. vesiculosus* individual with the Hve haplotype.

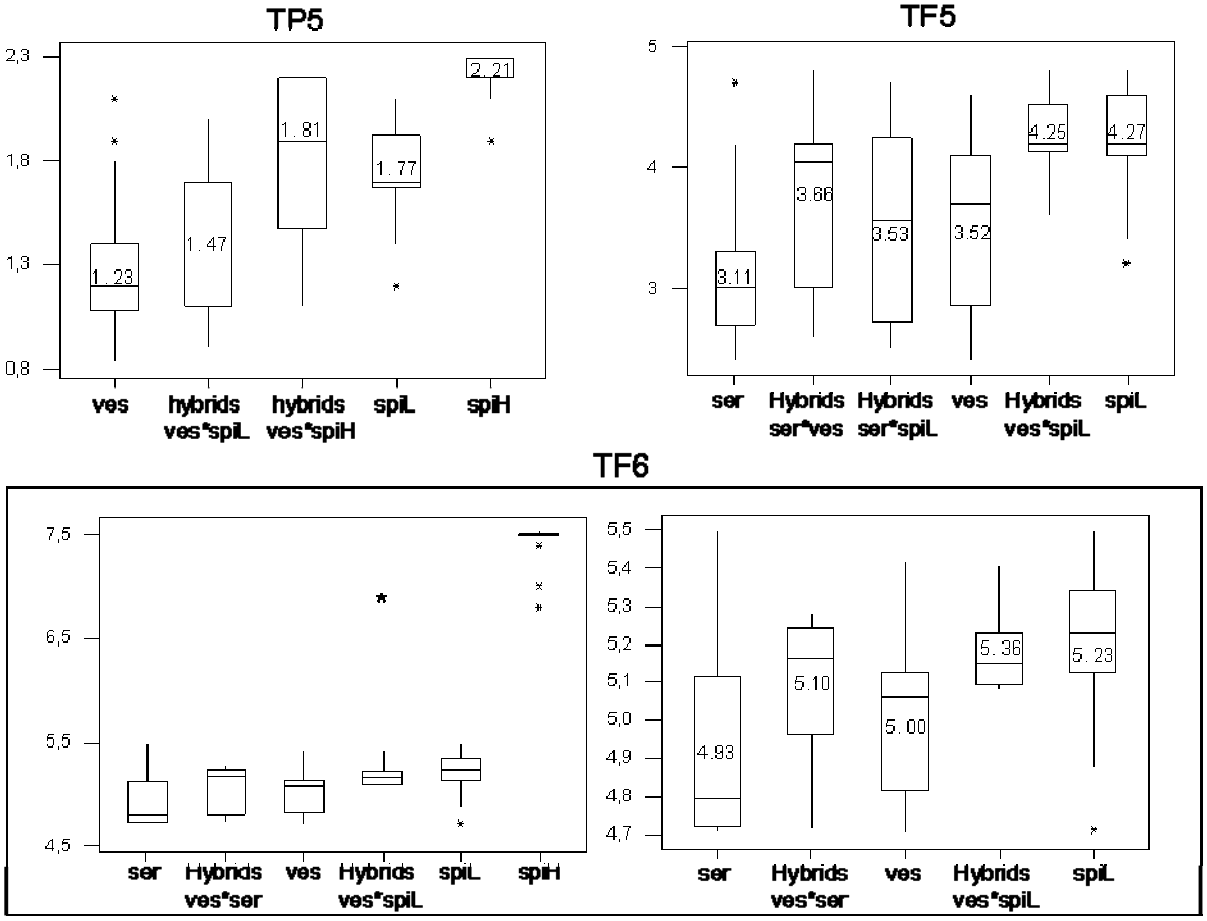


Figure 5: vertical distribution of taxa along the transects. data not available for TP6. TF6 is shown with and without spiH and the unusual value of Hybrids ves*spiL. Medians are represented with horizontal bars and mean values are indicated in the stat boxes.

Table 6: Frequency of haplotypes. A: Rubisco-spacer chloroplastic marker :rbc within the different clusters defined with nuclear genotype. B: psbD-rpl19 observed within each cluster and rbc-haplotype

A

		Haplotypes		
Nuclear genotype	Nb ind	*spiralis* (Hsp)	*vesiculosus* (Hve)	*serratus* (Hse)
F. spiralis high	18	1.00	0	0
F. spiralis low	47	1.00	0	0
F. vesiculosus	80	0.25	0.75	0
F. serratus	25	0.40	0	0.96
Hybrids ves*spiH	4	0.50	0.50	0
Hybrids ves*spiL	7	0.86	0.14	0
Hybrids ser*ves	9	0.22	0.67	0.11

B

		Haplotypes						
		Hsp				Hve		Hse
Nuclear genotypes	Nb ind	H1	H2	H4	H5	H1	H3	H1
F. spiralis high	17	1.00	0	0	0	0	0	0
F. spiralis low	43	0.72	0.26	0.02	0	0	0	0
F. vesiculosus	70	0.16	0	0	0.03	0.80	0.01	0
F. serratus	22	0.05	0.05	0	0	0	0	0.91
Hybrids ves*spiH	3	0.33	0	0	0	0.67	0	0
Hybrids ves*spiL	7	0.57	0.14	0.14	0	0.14	0	0
Hybrids ser*ves	9	0.22	0	0	0	0.67	0	0.11

iii. Correspondence genotype / phenotype

On the total of 1174 individuals observed (see table 1), 12% presented an intermediate phenotype, mainly a dioecious sexual phenotype associated with the absence of vesicle (92%). Typical morphotypes were strongly correlated with genetic cluster (table 7). However, the most conspicuous character, i.e. the absence/ presence of vesicles was not completely reliable as diagnostic for the species *F. vesiculosus* at these sites, since only 9.6% of the intermediate phenotypes were assigned as hybrids while 50% were actually assigned to the *F. vesiculosus* cluster. This result is mostly due to dioecious individuals not bearing vesicles (15% of the *F. vesiculosus* individuals exhibited an intermediate phenotype). On the other hand, the sexual phenotype appeared to be a good species indicator, 2.98% of *F. vesiculosus* were hermaphroditic and 2.59% of the *F. spiralis*-Low individuals were dioecious. Since the first analyses of sexual phenotypes were done by sampling two receptacles per individual for a given time, and for some individuals all receptacles were observed, the stability of sex was also checked within individuals among receptacles and through time. Only one hybrid individual in France was found to have 3 female receptacles among 29 hermaphrodite ones. In Portugal, three *F. vesiculosus* individuals showed one variant receptacle (2 males, 1

hermaphrodite) among a majority of female receptacles (30,51,22 respectively), whereas one *F. spiralis* individual had 10 female and 32 hermaphroditic receptacles. No temporal variation was observed during the nine-month survey of the individuals from the 2006 transect in France.

Table 7: Observation of the morphological characters according to the genotypes. For each genotype/phenotype combination, the first percentage indicated is the proportion of a given morphotype in each cluster and the second percentage is the proportion of a given genetic cluster in each morphotype (100% of individuals from the *F. spiralis* high cluster present the *spiralis* morph, but 17% of individuals presenting the spiralis morph belong to *F. spiralis* high cluster).

		Phenotypes					
Nuclear genotype	Nb ind	*spiralis* morphotype		*vesiculosus* morphotype		intermediate morphotype	
F. spiralis high	42	100.0%	17.36%	0.00%	0.00%	0.00%	7.11%
F. spiralis low	193	94.8%	75.62%	0.00%	0.00%	5.18%	32.66%
F. vesiculosus	299	1.3%	1.65%	83.61%	89.93%	15.05%	50.59%
Hybrids ves*spiH	12	25.00%	1.24%	66.67%	2.88%	8.33%	2.03%
Hybrids ves*spiL	45	22.22%	4.13%	44.44%	7.19%	33.33%	7.61%

iv. Genetic structure / breeding system

Genetic distances between all clusters of transects except *F. serratus* were visualised with a factorial correspondence analysis (FCA), which confirmed the occurence of the three clusters (Fig. 6). Indeed, the individuals of *F. spiralis* grouped according to their cluster, independently of geographic distance, while *F. vesiculosus* populations were distinguishable according to regions of origin (F_{ST} within region 0.006-0.02; F_{ST} between regions 0.11-0.17). Nevertheless, all F_{ST} values were significant, revealing a differentiation within species at a low spatio-temporal scale. F_{ST} between populations of *F. spiralis* were all highly significant whatever the pair considered (Table 8). F_{ST} between clusters were higher than within. The high value observed between spiLP5 and spiLP6 was due to L94 which was fixed or nearly fixed for different alleles (value drops to 0 when this locus is not considered). Differentiation between the two clusters of *F. spiralis* was again mainly due to loci L20 and L78. (Fig.7)

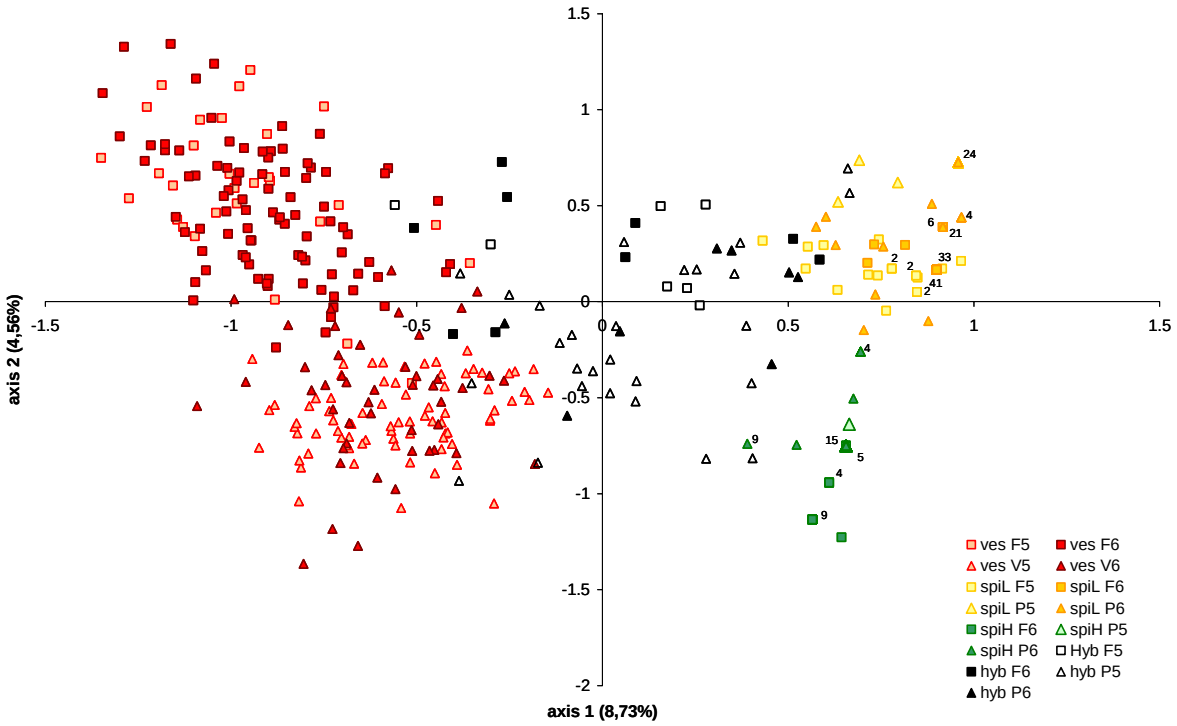


Figure 6: Factorial analysis on individuals of populations of *F. vesiculosus*, *F. spiralis* low, *F. spiralis* high and their hybrids in the four transects.

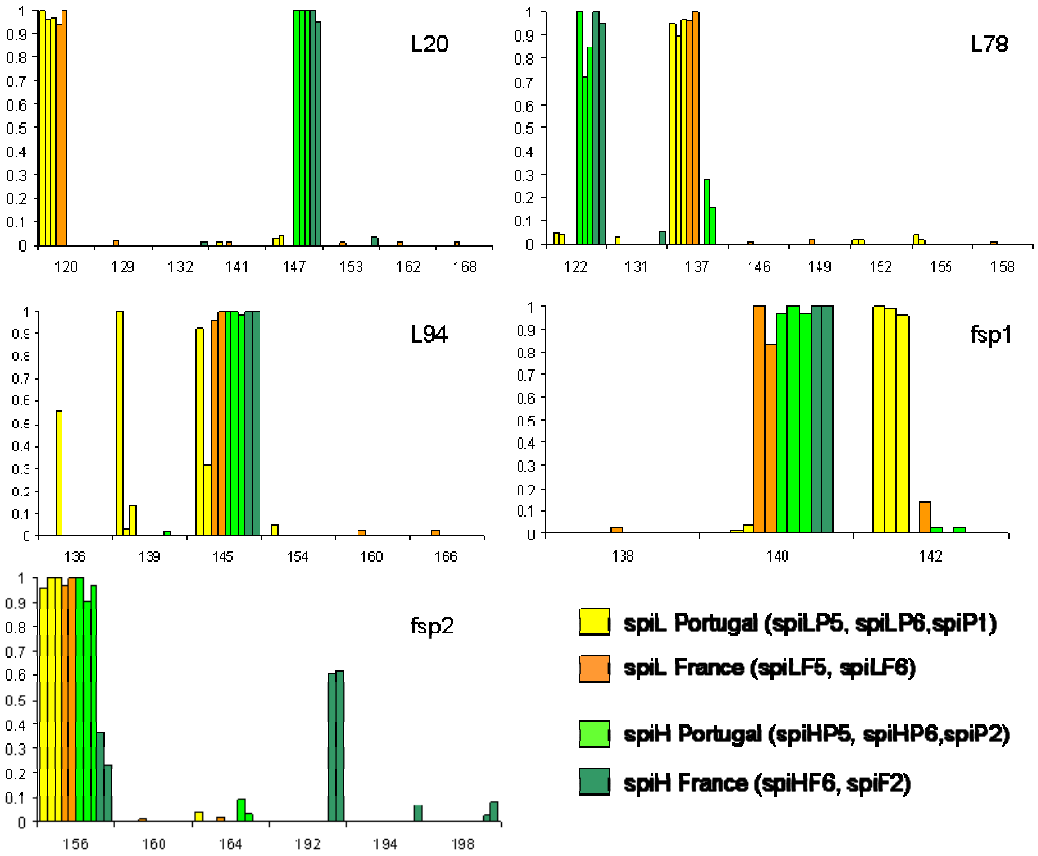


Figure 7: Allele frequency within *F. spiralis* populations from transects and parapatric quadrats. Loci L38 and L58 are not shown because they were monomorphic

Table 8: F_{ST} between populations of *F. spiralis* low and high between regions and between years.

F_{ST}	spiLP5	spiLF6	spiLF5	spiHP6	spiHP5	spiHF6
spiLP6	0.725***	0.653***	0.704***	0.819***	0.889***	0.863***
spiLP5		0.866***	0.865***	0.911***	0.965***	0.931***
spiLF6			0.065***	0.799***	0.888***	0.866***
spiLF5				0.769***	0.864***	0.847***
spiHP6					0.174***	0.408***
spiHP5						0.544***

Genetic structure of these sexually stable genetic clusters was investigated (Table 9) within the four transects. Whatever the population, observed heterozygosity (H_o) was at least one order of magnitude higher in *F. vesiculosus* than in both *F. spiralis* clusters. Within *F. vesiculosus*, genetic diversity was also higher in France than in Portugal as shown by H_o, by the mean number of alleles per locus and the number of private alleles

Table 9; genetic diversity within parental clusters defined in the four transects. VesP5: *F. vesiculosus* transect Portugal 2005 etc. N, number of individuals; H_E total expected heterozygosity (SE); H_O total observed heterozygosity (SE); P(0.95), proportion of polymorphic loci (where most common allele does not exceed 0.95) A, mean number of alleles per locus; SSA, species-specific (private) Allele; SSA (0.05); number of species-specific alleles at a frequency >0.05

Pop	*N*	*H_E*	*H_O*	*P(0.95)*	*A*	*SSA*	*SSA (>0,05)*	*SSA*	*SSA (>0,05)*
vesP5	77	0.563	0.482	1.00	5.3	10	4		
vesP6	21	0.611	0.503	1.00	4.9	8	5	36.0	5.0
vesF5	28	0.728	0.622	1.00	8.0	23	7		
vesF6	87	0.745	0.703	1.00	10.3	27	7		
spiLP5	27	0.025	0.011	0.14	1.4	1	1		
spiLP6	28	0.067	0.026	0.43	2.1	1	0	1.0	1.0
spiLF5	45	0.055	0.041	0.14	2.9	0	0		
spiLF6	48	0.044	0.006	0.14	1.4	0	0		
spiHP5	16	0.009	0.009	0.00	1.1	0	0		
spiHP6	16	0.082	0.018	0.29	1.3	0	0	1.0	0.0
spiHF6	19	0.071	0.038	0.14	1.3	1	0		
serF6	21	0.742	0.612	1	9.3	29	7	29.0	7.0

.

Multilocus F_{IS} values indicated high heterozygote deficits in all clusters (Table 10) suggesting inbreeding in *F. vesiculosus* populations. However, it was not due to the same loci. L20 was highly significantly positive in all *F. vesiculosus*. On the contrary, for L94 and fsp1, F_{IS} was not significant in almost all *F. vesiculosus* (except for vesP6: F_{IS} = 0.073, P= 0.05). L58 and fsp2 presented different levels of F_{IS} in *F. vesiculosus*, according to the region and finally, L38 and L78 did not present any clear pattern. In *F. spiralis*, F_{IS} was highly positive, when it could be calculated.

Table 10: Locus by population *F*is (W&C) estimates

	F_{IS}							
	over all loci	L20	L38	L58	L78	L94	fsp2	fsp1
vesP5	0.149***	0.346***	0.159*	-0.205*	0.034	0.059	0.646***	-0.003
vesP6	0.200***	0.623***	-0.204	-0.268*	0.253***	0.061	0.580***	0.073*
vesF5	0.162***	0.186***	0.165*	0.111	0.289***	0.143	0.134*	0.083
vesF6	0.063**	0.139***	0.004	0.101*	-0.036	0.03	0.159*	0.051
spiLP5	0.595***	------	------	------	0.320***	------	1***	------
spiLP6	0.632***	0.662***	------	------	0.585***	0.792***	------	0
spiLF5	0.265***	-0.031	-0.011	------	0.747***	0.326*	0.326*	------
spiLF6	0.867***	------	------	0	------	------	------	0.929***
spiHP5	0	------	------	------	------	------	------	0
spiHP6	0.795**	------	------	------	0.854***	------	0.651***	------
spiHF6	0.492***	------	------	------	------	------	0.492***	------
serF6	0.198***	0.067	0.048	0.235***	0.429***	0.271*	-0.143	0.295

v. Spatial autocorrelation.

The high magnitude of slope for vesP6, compared with other *F. vesiculosus* populations (though almost similar to F6), suggested that individuals were not randomly distributed in some cases at least, suggesting restricted egg dispersal. For *F. spiralis*, the lack of polymorphism exhibited by several loci, avoided their use for fine scale analysis. Four loci were informative for spiP6 and spiF5, three for spiP6 and only two could be used for spiF6. However, the significant negative slope for spiLF5 as well as for spiF6 by the chloroplastic locus psbD-rpl19 (fig. 8) suggested restricted dispersal also in *F. spiralis* at least in some cases. The cases where the slope was not significantly different from zero may represent no departure from random genotype distribution in several areas or lack of power to detect it, given the low number of individuals per class and of loci.

Table 11: Slopes of spatial autocorrelogram over all loci for each cluster and each transect.

	TP5	TP6	TF5	TF6
F.spiralis high	-0.0004 ns	0.0010 ns		
F.spiralis low	-0.0002 ns	-0.0020 ns	-0.0080 (<0.001)	-0.0007 ns
F. vesiculosus	-0.0006 (0.025)	-0.0060 (<0.001)	0.0001 ns	-0.0002 ns

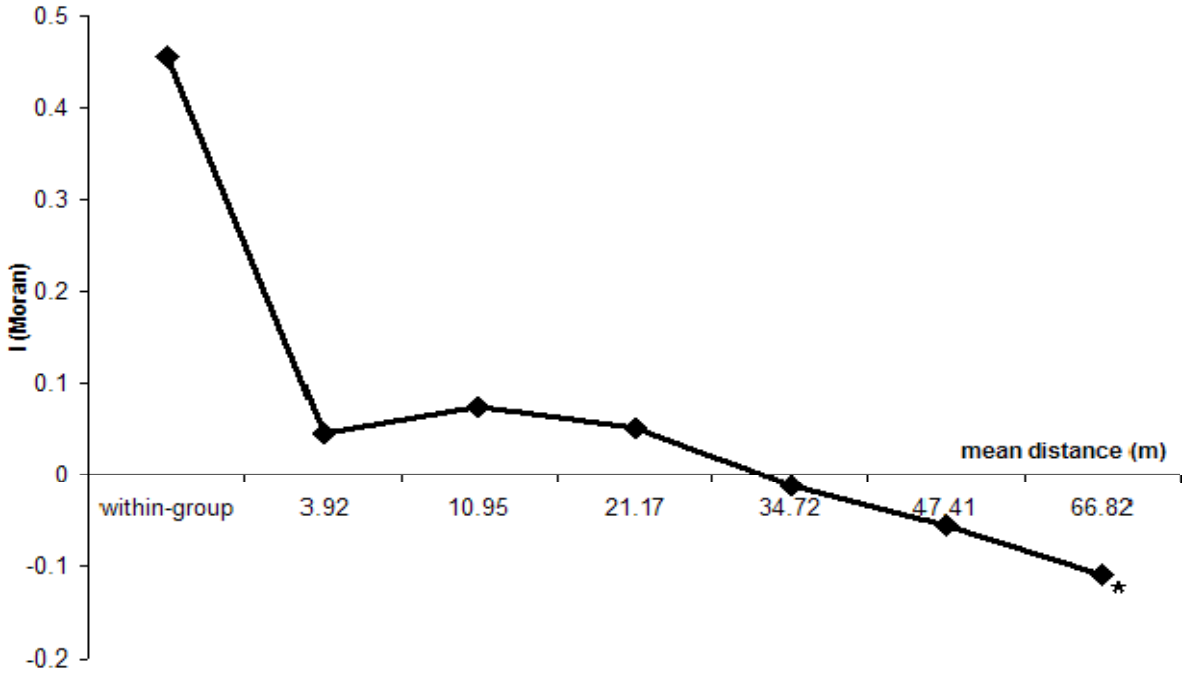


Figure 8: autocorrelogram of *F. spiralis*-Low individuals from TF6 (transect France 2006) obtained from the chloroplastic marker psbD-rpl19. slope = 0.003, p=0.017

6.4.2 Phenology

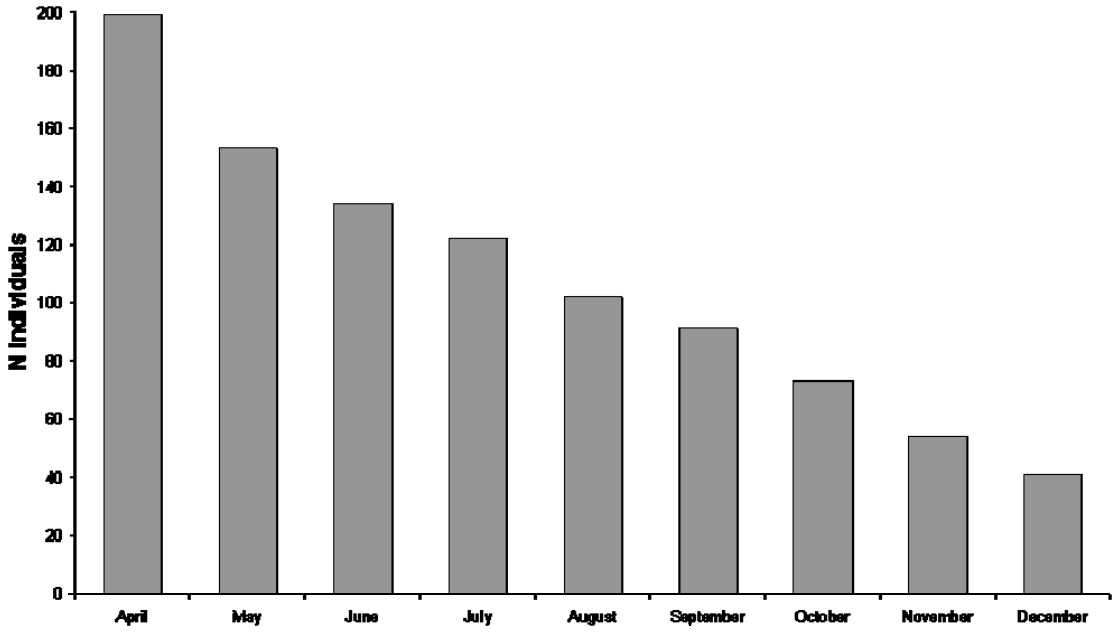


Figure 9: Number of labelled individuals found each month during the phenological survey

Close relationship may exist between breeding system, hybridization and phenology, thus individuals from TF6 (Transect France 2006) were surveyed monthly during 9 months. Individuals were regularly lost, during the survey (Fig.9) due to mortality or manipulation, but we suspect it was mainly due to water motion. Most of individuals were attached to rocks in the sand, but sometimes rocks were small and not strongly fixed in the substratum, so they could derive. No significant difference was detected in the total duration of maturity according to gender. Males were mature during 4.0 (± 2.8) months, females 3.3 (± 2.1) months and hermaphrodites 3.6 (± 2.3) months. However, the pattern of maturity seemed to be quite different among genders (fig. 10). Indeed, when comparing each month the proportions of the four following states of maturity for each gender, ie immaturity, early maturity (gender could be seen but receptacles were not ready to release) maturity and end of maturity (receptacles getting rooted), we found out that a majority of hermaphrodite were mature during the nine months of survey, that most of males were mature during spring and then in winter, while they were mostly immature during summer and finally less synchronous than females. The majority of females were mature during spring (100% in May) then, in July, 80% became immature, suggesting that major events of release had occurred during

June. Like males, the remaining females became mature again in January. This synchronicity of female had an effect on sex-ratio estimation (fig. 11). Indeed, the only significant departure from 1:1 ratio occurred in July, when females became immature. This effect of female maturity on the calculation of sex-ratio is also seen vertically (Fig 12). The proportion of males over the total number of individuals did not vary; the difference between the lower and the upper part of the shore was the proportion of females and immatures. Sex ratio was calculated without regard to the period of maturity. However, due to the loss of individuals each month, individuals reaching maturity later may have be lost before their sexual phenotype had been observed.

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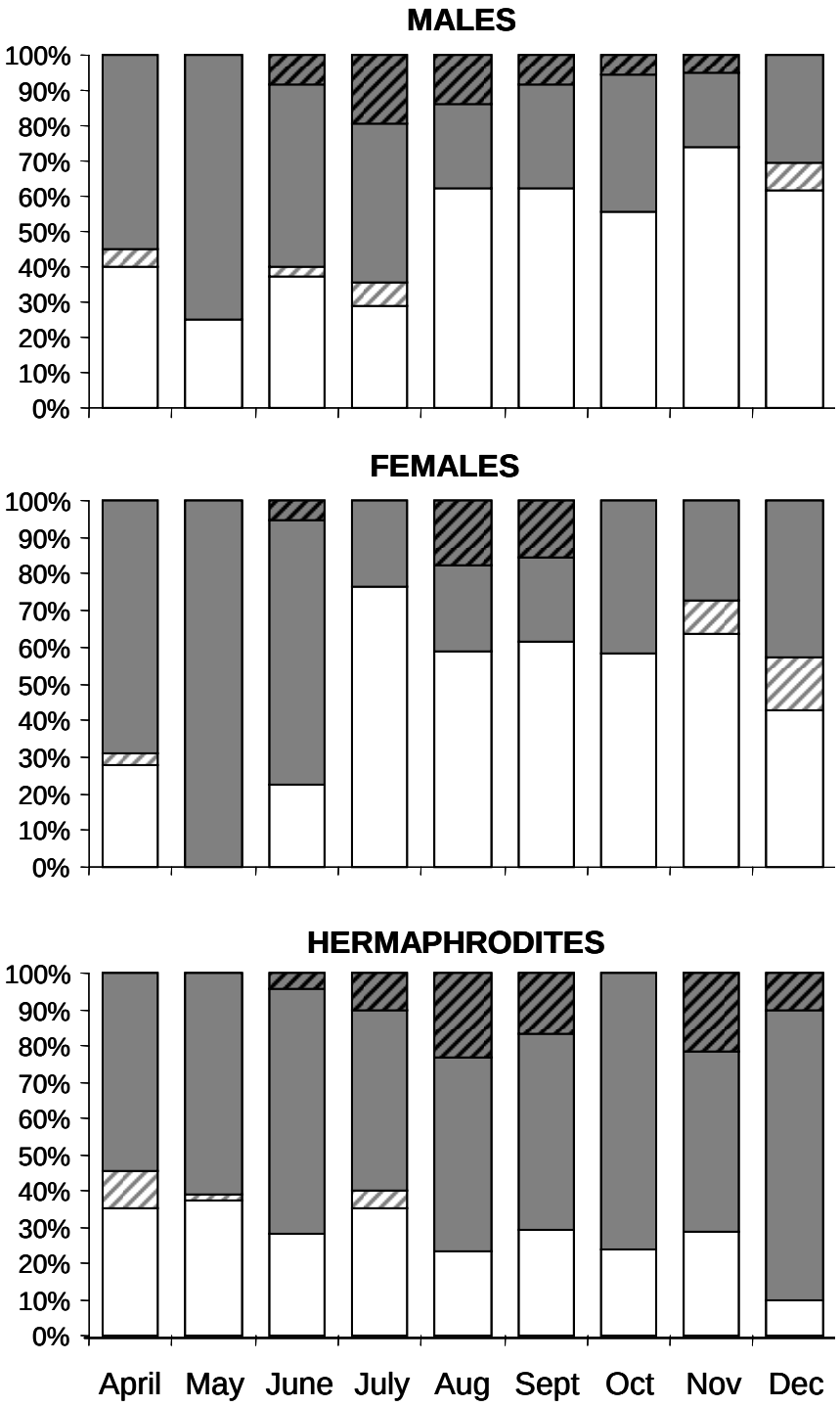

Figure 10: Proportion of individuals at four different maturity states. Immature (white), early maturity (white/grey hatched) mature (grey) and end of maturity (grey/black hatched)

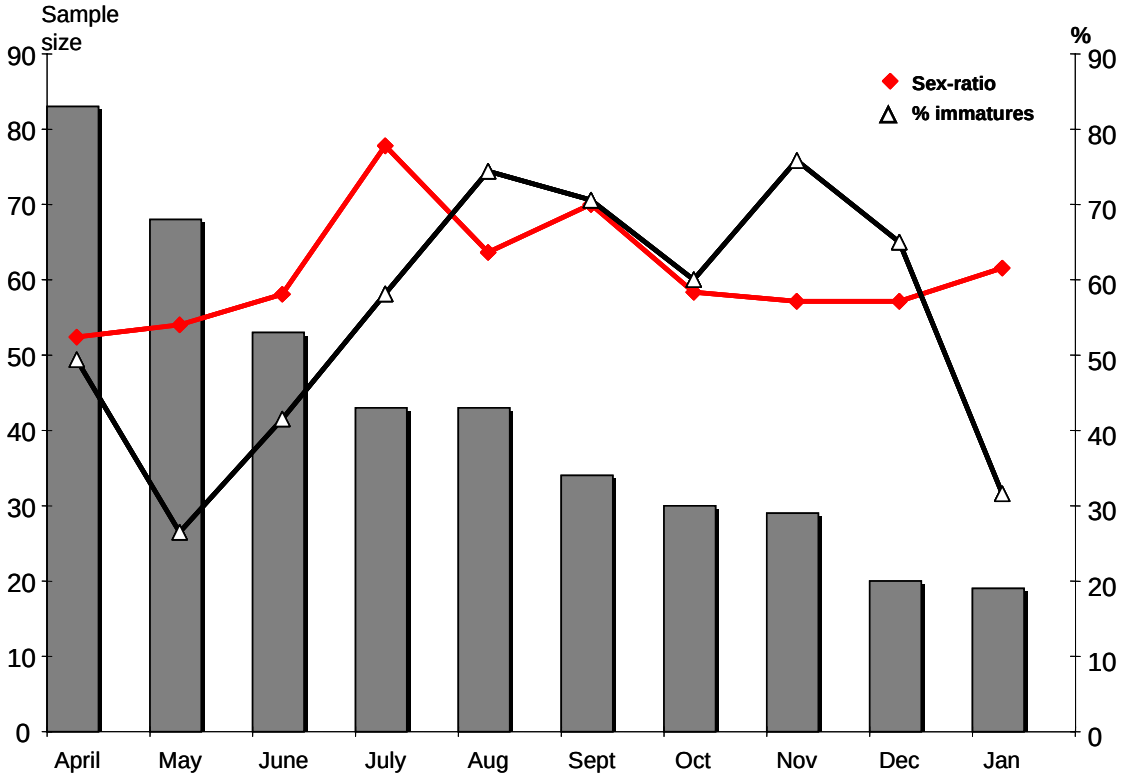

Figure 11: Evolution of the sex-ratio (proportion of males over the total number of individuals) in *F. vesiculosus* in relation with the proportion of immature individuals in the population and with the number of individuals analysed. Sample size is represented by grey bars.

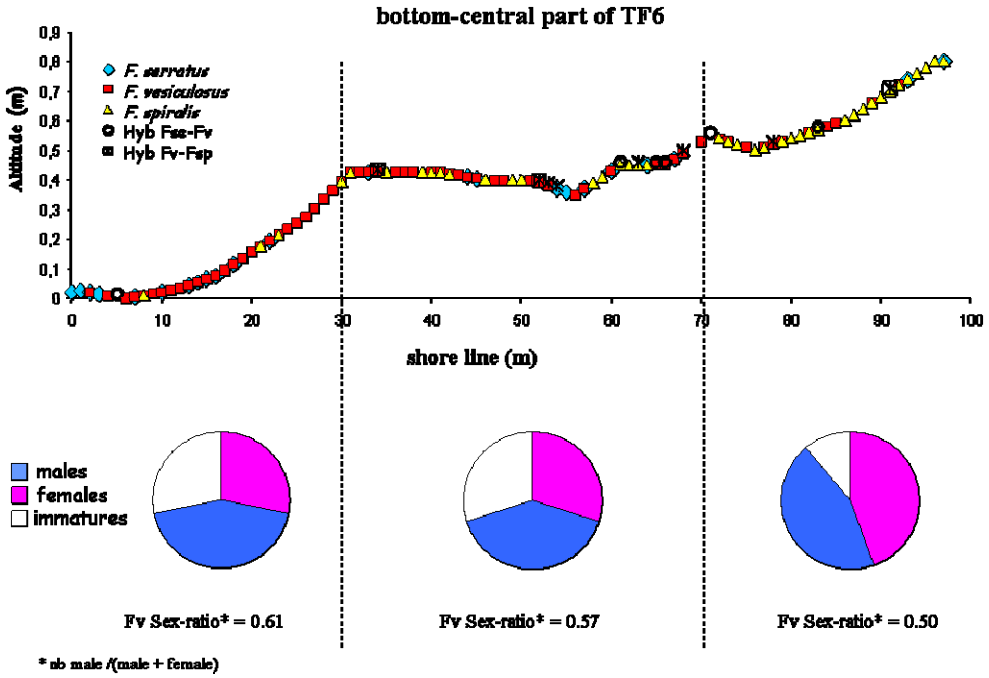

Figure 12: sex-ratio according to the proportion of immatures and the position on the shore in the transect France 2006

6.5 Discussion

6.5.1 Number of genetic clusters.

i. F. spiralis is composed of two clusters

All results from genetic (F_{ST}, assignment and CA) and ecological (position on transect) analyses are consistent with the occurrence of two different entities within *F. spiralis*. This genetic data could confirm Pottas' works (Pottas, 2006) who identified two stable morphotypes of *F. spiralis* along the North Yorkshire shore (England). Although undistinguishable on the basis of allozymes, these two morphotypes were persistent through seasons and development stages and were only associated with the height on the shore. The genetic characterisation of these morphotypes with the microsatellites used in this study should allow to determine if they correspond to the *F. spiralis*-High and *F. spiralis*-Low entities revealed in this study.

This occurrence of two distinct genetic entities has been previously detected, although not suspected by Engel et al (2005) who observed strong allele frequency differences for loci L20 and L78 between *F. spiralis* from parapatric and sympatric quadrats in Viana do Castelo. Perrin et al (2007) also observed numerous linkage disequilibria over loci in their studied populations of *F. spiralis* and interpreted it, as a clue of high selfing rate, supported by high F_{IS} value as well (*NB*: these two authors did not use the same nomenclature for 3 loci. For correspondence: add 19, 16 and 18 bp to alleles defined by Engel at locus L38, L78 and L94 respectively, in this study, we used Engel's nomenclature (Engel et al., 2003). In a previous study of populations of Brittany (Billard et al., 2005a) we already reported the diagnostic character of L78 between *F. spiralis* and *F. vesiculosus*, this affirmation must be now qualified: from our data and previous results (Engel et al, 2005; Perrin et al., 2007): L78 appears to be highly discriminant for *F. spiralis*-High (allele 122) and for *F. spiralis*-Low (allele 137), while L20 seems diagnostic for *F. spiralis*-Low (allele 120). It is interesting to notice that individuals sampled in 2002 (Engel et al, 2005), in 2003 (Perrin et al. in prep), and in this study, often are fixed or nearly fixed within each sample for particular combinations of alleles from locus L20 and L78, suggesting very local spatial variation and very restricted gamete dispersal. Taking into account the existence of two different populations, F_{IS} in Engel et al (2005) could have been artificially increased by Wahlund effect, however in Viana Pavia et al., this effect could only have affected the quadrats where the two types occurred mixed, and in France it could not have affected them because the type F. spiralis-Low was never present in the French samples of Engel et al. (2005), and thus it could not have affected all F_{IS}

values of the study. Furthermore, the low degree of polymorphism showed by loci and the observed value of F_{IS} within each cluster still support the presumption of high selfing rate. On the other hand, hybridization between the two forms could not be an alternative hypothesis to those emitted by Perrin et al (2007) to explain observed heterozygote excess for Fsp2 in marginal populations of *F. spiralis* in Southern Portugal, because the type *F. spiralis*-High (FspiH) has never been found in these populations, using L78 as diagnostic between the two (Perrin et al. in prep).

The fact that the two populations of FspiH (Brittany and Portugal) group together in the correspondence analysis (Fig.6) rather than with the FspiL (*F. spiralis*-LOW) population from the same site, as well as the occurrence of two distinct morphotypes along the European coast (although it remains to be checked that the morphotypes observed by Pottas (2006) correspond to the different genetic entities) suggests that they are different species. A comparison of *F. spiralis* genotypes from their entire biogeographic distribution reveals that these are mainly geographically segregated entities that co-occur in Portugal and France (Perrin et al. in prep) and that "hybrids" between the two types exist, although rare (Perrin et al. in prep). However, when looking at the allele frequency, we can see that the difference is only due to the loci L20 and L78. Thus, local adaptation and selection cannot be rejected and on the contrary adaptation to desiccation possibly interacting with competition driving the emergence of a species complex would be an explanation for genetic divergence between these two clusters. Current studies on desiccation-induced stress conducted for *F. vesiculosus* and *F. serratus* (Lago-Lestón, Pearson, Coyer) might provide new tools to answer this question. The neutral evolution of loci L20 and L78 may thus also be questionable. It is possible that these two loci are linked to a selected gene and thus, that they evolve under selection, just by hitch-hiking effect increasing F_{ST} between populations (Beaumont, 2005; Beaumont & Balding, 2004; Nielsen et al., 2006).

ii. Phenotypic polymorphism in F. vesiculosus.

Contrary to *F. spiralis*, *F. vesiculosus* in the two sampled regions appeared to be a true homogeneous genetic entity but phenotypic polymorphism was observed. Indeed, 15% of *F. vesiculosus* did have an intermediate phenotype in what concerns the character presence/absence of vesicles. Several forms of *F. vesiculosus* without vesicles have been reported to occur in different habitats (e.g., Burrows and Lodge 1951, Ardré 1970, Gómez Garreta 2001), particularly some unattached forms inside some estuaries and other forms on exposed rocky shores, whereas in sheltered rocky shores *F. vesiculosus* populations tend to

have vesicles, and genotyping has revealed that presence/absence of this character does not imply genetically distinct populations (Daguin et al. in prep). However since this is a character that tends to vary depending on the site, we investigated whether this could still be a useful phenotypic character to distinguish the species *F. vesiculosus* and *F. spiralis* within the particular sites of this study. This was not the case however, because we found that within each site individuals with and without vesicles occurred side by side. Particularly, in Portugal, the occasional absence of vesicles caused misidentification of a *F. vesiculosus* population as a sympatric area between *F. vesiculosus* and *F. spiralis*. Moreover, during the nine-month survey, we observed that the presence of vesicle was quite inconsistent; they were not present in juveniles and often disappeared with grazing or when individuals were damaged. Thus, in the absence of vesicles the only reliable criterion to distinguish species is the sexual phenotype.

6.5.2 Diagnostic marker in Chloroplast

i. Rubisco marker

As explained in Billard et al (submitted): in parapatric populations, Hsp and Hve were strictly associated with *F. spiralis* and F. *vesiculosus* species respectively. The maternal inheritance of organelles in *Fucus* (Brawley et al 1976, Coyer et al 2002) allows tracing gene flow associated with eggs. Thus, the identification of three species-specific haplotypes for *F. vesiculosus*, *F. spiralis* (H and L) and *F. serratus* respectively, in combination with the large panel of nuclear microsatellites now available for *Fucus* species (Coyer et al 2002, Engel et al 2003, Wallace et al 2005, Perrin et al 2007) should provide now a simple and powerful tool for studying interspecific gene flow between these species, allowing to estimate the orientation as well as the extent of hybridization between them.

ii. psbD-rpl19

This second chloroplastic marker appeared to be more polymorphic in *F. spiralis* Low than in each other cluster, although only in France because it was completely fixed in all Portuguese populations. Haplotype H1 was highly dominant in all clusters. When psbD-rpl19 haplotypes were compared with rubisco haplotypes; they revealed that four psbD-rpl19 haplotypes (H1, H2, H4 and H5) were associated with Hsp haplotype, whereas Hve and Hse were almost exclusively associated with H1. The polymorphism attached to the Hsp

haplotype supports results showed by phylogeny (Billard et al, submitted). Indeed, it suggests that mutations had more time to occur in *F. spiralis* chloroplast than in the other species, thus that *F. spiralis* should be ancestral to the other species. This is also consistent with Coyer et al (2006) scenario of a *F. spiralis* ancestor for lineage 2 (*F. vesiculosus*, *F. spiralis*, *F. ceranoides*, *F. virsoides*).

6.5.3 Hybridization

i. Extent and orientation of hybridization

Hybridization seems to occur only over very short geographic distances, as shown by the different hybridization rates observed in parapatry and sympatry, which confirm previous results by Engel et al (2005). Now taking into account the two clusters of *F. spiralis*, we observed that number of hybrids ves*spiL was twice as important as the number of hybrids ves*spiH. Since spiH are located higher on the shore (Fig. 5), it may confirm that hybridization occurs at very low spatial scale and that only a small vertical distance can limit gene flow.

Interestingly, in France we observed that hybridization occurred between *F. vesiculosus* and *F. serratus* and between *F. serratus* and *F. spiralis* as well. Hybrids ser*ves were even more frequent than hybrids ves*spi, contradicting the observation by Coyer et al (in press) that hybridization in *Fucus* is limited to sexually contrasting sister species. It may also occur between the two lineages. Nevertheless, the absence of *F. serratus* haplotypes in the other species and the unique *F. spiralis* haplotype in *F. serratus* suggest that hybrids are sterile or counter-selected. That *F. serratus* and *F. vesiculosu*s can hybridize is not so surprising from an ecological standpoint; both species are dioecious, with external fertilization and highly mixed on the shore, at least in our study site of Brittany. However, it is more surprising from the phylogenetic standpoint, since relationships between these species are much more distant. Hybrids between *F. vesiculosus* and *F. serratus* had been experimentally produced (e.g., Bolwell et al. 1977) but up to now not confirmed to occur in nature using molecular markers. The observation of hybridization between *F. vesiculosus* and *F. serratus* raises the points that:

- divergence within the Fucus genus must be too recent to completely avoid crosses between the two lineages

- genetic incompatibility must not be sufficient to create barriers to maintain genome integrity in sister species. Other factors may act against hybridization, like environmental factors and/or mating systems.

Indeed, mating systems seem to play an important role, since hybridization appears to be asymmetric. According to the observed rubisco-spacer haplotypes in the French site, interspecific crosses were due to sperm from *F. vesiculosus* males and eggs from *F. spiralis* hermaphrodites in one case and to sperm from *F. serratus* and eggs from *F. vesiculosus* in the other case. In both dioecious species, the sperm:egg ratio is ten times higher than in the hermaphroditic one (Vernet & Harper, 1980 Billard et al., 2005b). The difference in amount of sperm could explain the orientation of hybridization in that case. Moreover, in Portugal and in France, hybrids ves*spi (L and H) were found between parental species, since eggs have negative buoyancy, they may sink near the "mother" individual or lower on the shore, while male gametes may be washed more easily and carried at a higher level on the shore by water with the tide. This could explain the hybridization pattern observed for *F. serratus* and *F. vesiculosus* as well. However, this hypothesis is not completely satisfying concerning these later species, because in the study site, they were found highly mixed (Fig. 5). Differences in the timing of release of gametes during the tidal cycle could also result in differential hybridization if all eggs of one species are fertilized before the second one starts releasing gametes. Indeed, in corals small differences in spawning peak times during the day are sufficient to avoid hybridization (Levitan et al., 2004). Another explanation could be selection against hybrids with a *F. serratus* chloroplast as suggested by Coyer et al (in press) from their *F. serratus* * *F. evanescens* hybridization study. Yet another explanation might be differences in gamete compatibility and consequent fertilizability (i.e., the proportion of egg:sperm collisions that originate a fertilization) between sister species, whereby species that encounter more frequently sperm limitation conditions (possibly higher towards the top of the shore, given more limiting conditions) might tend to have gamete recognition proteins that, while increasing fertilizability at the intra-specific level result also in eggs that are more easily fertilized by non-specific sperm. Such a trade-off between increasing fertilization success versus preventing hybridization has been observed in sea urchins (Levitan, 2002).

The last remarkable point is the difference in hybridization rates we observed between TP5, the transect sampled in Portugal (at a site in Viana in 2005), and the three other transects, TP6, TF5 and TF6 (Table 5). Hybridization rate between ves and spiL is more than twice as important in TP5 (12.7%) than in the other transects (5.6%). Moreover, this difference is even higher between quadrats in this region (Table 4). A bias induced by the

determination of hybrids is rejected because French sympatric quadrats, in which very few *F. serratus* were found, presented even lower hybridization rate. A possible explanation for the difference between France and Portugal could be that Viana do Castelo, the Portuguese site, is near the southern limit of sympatry between *F. spiralis* and *F. vesiculosus* (Engel et al., 2005). Beyond the region of North Portugal, *F. vesiculosus* is restricted to estuarine habitats (Ladah et al., 2003). Northern Portugal can thus be considered as a marginal habitat for *F. vesiculosus*, favouring hybridization (Herlihy and Eckert, 2005). However, populations of *F. vesiculosus* from Viana do not appear sparse or less fit, and species areas seem to be more defined and less overlapping in Viana than in France. Thus, instead of a weaker hybridization barrier in Portugal, an alternative hypothesis could be a stronger barrier in France as a reinforcement of selection against hybridization (Noor, 1999; Turelli et al., 2001; Wallace, 1889, Smadja and Butlin, 2006). Yet another possibility is that being located at the boundary of the allopatric distribution range between these species (because further South they occur but never in sympatry) implies that higher hybridization susceptibility could be due to gene flow from nearby allopatric populations which have evolved without the need for investment into barriers against hybridization (Noor, 1999 . Nevertheless, none of these hypotheses can explain why hybridization rate is so different between the two sites in Portugal, one sampled in Viana South in 2005 and one sampled in Viana North in 2006 (Fisher exact test P=0.02). These differences are less likely to be due to temporal rather than spatial effects because these individuals are perennial, and thus populations are not expected to change their genetic composition significantly from year to year. The two sampled sites have slightly different exposures and higher exposure can act in preventing spawning events. Fucus release their gametes in calm conditions and synchronously (see for review Pearson and Serrão 2006), but if hydrodynamic conditions cause spawning to be delayed, this may lead to gametes aging and being released under physiological conditions in which hybridization is more likely, as was suggested by Berndt et al (2002). This is possible because *Fucus vesiculosus* and *Fucus spiralis* have the same reproductive season and release their eggs synchronously on the same days of the month each month (Monteiro et al. in prep, Pearson and Serrão, 2006).

ii. Phenology and Breeding system

Temporal reproductive isolation between closely adjacent populations is well known as a speciation factor. In pollinated flowering plants, it has long been reported as a mean of sympatric speciation (Ollerton, 2005; Pascarella, 2007). Fertilization success in Fucus is highly dependent on the synchronicity of gamete release (Pearson, 1996; Serrão, 1996

Pearson and Serrão 2006). The patterns of individual maturity should reflect this synchronicity. During the survey, we observed that individuals of both species were perennial with several maturity periods followed by decay and death of semelparous branches. Like in *F. distichus* Ang, 1992 new receptacles form while old ones are dying. Thus, individuals are fertile during several months of the year. Moreover, all individuals were not mature at the same time resulting on a continuous fertility of the species throughout the survey period. Sexual phenotypes of successive receptacles within individuals were stable, supporting the hypothesis of genetic control of mating system rather than environmental. Pattern of maturity period was however quite different according to gender. Indeed, females appeared to be more synchronous than males and hermaphrodites (Fig. 10) which had a strong effect on the estimation of sex-ratio (Fig.11). The synchronicity of females is consistent with the observation of a synchronous gamete release in a species with external fertilization. However males appear to be still mature in July, while most of females are immature, suggesting that they may be likely to release sperm even in the absence of mature females. On the other hand, hermaphrodites do not show any strong peak of maturity or vegetative period. This suggests that they should reproduce during all the year. In July, the biased sex-ratio towards males in *F. vesiculosus* and the presence of reproductive *F. spiralis* may favour hybridization between these two species and could be additional explanation to the orientation of hybridization. It could be interesting to test if hybrids are more frequently formed during the months when males are in excess.

Discrepancy in phenology was also observed between the lower and upper part of shore. Since sexual phenotype of individuals was checked every time they were found, sex-ratios presented in figure 12 represent individuals' gender without regard to their maturity period. However, due to the loss of individuals each month, the longer an individual stayed immature, the more chance it has to be lost before being sexually identified. The loss of individuals was homogeneous between the different parts of the transect, thus the sex-ratio may be biased by maturity discrepancy between the different parts of the transect and by differential loss of individuals along the shore. Sex-ratio is not different from 1 to 1 in most parts of the transect, it decreases with height on the shore. Proportion of males does not vary greatly, once again sex-ratio is affected by the relative proportion of females and immatures: females were less mature in the lower part of the transect. This may reflect a temporal discrepancy in the maturity of females from lower and higher part of the shore, which could limit vertical gene flow. Unexpectedly, temporal discrepancy seems to favour inter-specific crosses but limit vertical gene flow.

iii. Breeding system and dispersal

In addition high values of heterozygote deficiency indicate that spatial limitation to gene flow may also occur. Selfing has long been reported in *F. spiralis*, but it had not been addressed separately for *F. spiralis*-Low and *F. spiralis*-High. Here, heterozygote deficiency is confirmed in both clusters by high F_{IS} and very low polymorphism exhibited. Moreover, in this species egg dispersal may be very low, as suggested by the significant slope of spatial autocorrelogram based on the analysis of chloroplast marker psbD-rpl19. This result is found in only one case (TF5) when nuclear markers are analysed, suggesting either the sperm may disperse more randomly, either that dispersal scale is less than 1m and we can't detect it with our sampling design. Moreover, power of analysis may be reduced by the low polymorphism of loci in this species. Within *F. vesiculosus*, F_{IS} values also suggest inbreeding. Analysis of spatial autocorrelation gives different results in Portugal, where close individuals are more related, and in France where individuals seem to be randomly distributed. Once again, we can hypothesise that the sampling design missed the real dispersal scale; however another hypothesis might be the drift of individuals in the French site. Indeed, individuals grow on rocks buried in sand, but water motion can remove them and carry rock plus Fucus several meters away. In both species, high inbreeding (selfing) and low dispersal are thus suspected.

In this study, three different cases of hybridization and barriers to hybridization could be observed at a hundred meter distance scale:

1) between two dioecious species with apparently strong selection against hybrids, which suggest that genetic incompatibilities may have occurred
2) between two species with contrasting mating systems which appear to be maintained essentially by the evolution of life history traits under diverging selective pressure
3) between two clusters within the same species possibly in the speciation process caused by local adaptation to physical conditions and/or competitive exclusion and accelerated by the high selfing rate in this species.

Different ecological advantages conferred by dioecy and hermaphroditism may be the main mechanism maintaining genome integrity between *F. vesiculosus* and *F. spiralis,* as seen in an aquatic flowering plant (Dorken, 2003). It can be supposed that selfing was promoted in the higher part of the shore because of desiccation selection and the necessity of

maintaining co-adapted genes, since two microsatellite loci seem to be under selection while reallocation of resource towards male function and necessity to avoid inbreeding depression in a competitive habitat may have lead to dioecy lower in the shore.

Despite the unique opportunity to study hybridization at different taxonomic and ecological contrained levels, one case is missing: two closely related, dioecious species. Thus, it would be very interesting to conduct the same kind of analysis on the two species *F. vesiculosus* and *F. ceranoides*. On the other hand, *F. spiralis* L and H appear to be a very valuable model to study desiccation stress.

6.6 Acknowledgements

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CONCLUSION & PERSPECTIVES

7 CONCLUSION & PERSPECTIVES

Our studies, aiming at studying the evolution of reproductive systems in *Fucus*, as well as their implication in the processes of speciation and hybridization, can be summarized in some main results obtained by three combined approaches: phylogeny, population genetics and biology.

7.1 Phylogenetic approaches

7.1.1 Development cytoplasmic markers

At the beginning of this thesis, the need to clarify phylogenetic relationships between species of the genus *Fucus* on one hand and the intention to analyse the orientation of interspecific crosses suspected between the two species *F. vesiculosus* and *F. spiralis* on the other hand, have lead to the search for new markers of cytoplasmic DNA. Thus, these markers were looked for in the two organelles present in photosynthetic eukaryotes, the mitochondria and the chloroplasts. We focused our research on intergenic areas which are supposed to be variable enough (Gielly & Taberlet, 1994) to allow their use at the generic, or even specific level. The interesting characteristics of this type of markers are their haploid nature, their uniparental transmission, the absence of recombination and their supposed neutrality. Nevertheless, Bazin et al. (2006) highlighted the problem of the effect of selection on mitochondrial markers traditionally used in animals.

Within the mitochondria, we developed seven markers of which, three were easily amplified in the genus *Fucus*, however, they did not allow separating the three species (article 5, Engel et al, in press). The conservation of these markers among the different Laminariales families and Fucales species shows their potential great usefulness for phylogenetic studies. The higher polymorphism exhibited within Laminariales demonstrated that they are highly efficient markers for phylogeographic studies, like in the introduced species, *Undaria pinnatifida* (Voisin et al., 2005). Indeed, the use of two of these markers allowed identifying the main introduction vectors in Europe. Furthermore, the existence of two cryptic species in

the Laminariales *Lessonia nigrescens* has been shown along the Chilean coasts (Tellier et al., in prep).

7.1.2 Evolution of dioecy from hermaphroditism in *Fucus*

Concerning the chloroplast compartment, the development and use of three chloroplast markers within the Fucaceae family enabled to better resolve phylogenetic relationships and to show the probable independent evolution of dioecy from hermaphroditism among the different genera composing this family (Article 1, Billard et al., in prep). This result is concordant with theoretical models and observations performed in higher plants (Charlesworth, 1999; Desfeux et al., 1996). Moreover, among these markers, the Rubisco spacer was found to be diagnostic between the species *F. spiralis* and *F. vesiculosus* by the simple method of PCR-RFLP. However, this marker does not permit to resolve the totality of relationships between species composing lineage 2 of *Fucus*, particularly the status of *F. ceranoides*.

7.2 Population genetic approaches

7.2.1 Genetic barriers within the species complex *Fucus vesiculosus /spiralis / ceranoides*

Since the different phylogenetic analyses could not resolve the relationships between *F. vesiculosus*, *F. spiralis* and *F. ceranoides*, we used microsatellites, gene flow markers, for the population analysis of these three species at the regional scale of Brittany. We showed that reproductive isolation exists even if it is not complete (shared alleles between taxa, article 2 Billard et al. 2005a). Moreover, our results suggest, for locus 78, the existence of a specific allele in *F. spiralis* populations, a least in Brittany. This result was then refined.

7.2.2 Two divergent entities within *F. spiralis*

The most unexpected result of our study was the finding of two divergent genetic entities within *F. spiralis*: one found in the upper part of the distribution area of *F. spiralis* and the second one lower. This genetic divergence, showed with microsatellite markers, is observed in Portugal as well as in France and could result from a differential adaptation to

desiccation stress (see the review of Lexer & Fay, 2005). The difference between these two entities is mainly due to two loci: L20 and L78. This leads us to review the conclusions of the regional scale study performed in Brittany (Article 2, Billard et al., 2005a). Locus L78 still appears diagnostic for *F. spiralis*, it must now be precised that this locus has a specific allele for each of the two entities, high and low. The locus L20 also seems to be highly discriminant for *F. spiralis* low. These genetic results support morphological studies done by Pottas (2006) in England.

7.2.3 The shore as a model of hybrid zone in *Fucus*

The second unexpected result of this work was the hybridization shown not only between *F. vesiculosus* and *F. spiralis* low, but also between all *Fucus* species when individuals are in contact on the shore, it is to say: the two entities of *F. spiralis*, *F. vesiculosus* and *F. serratus*. With the analysis of cytoplasmic markers, we could determine that, like in the other lineage of *Fucus* (Coyer et al, in press), crosses are mainly due to the sperm of the dioecious species fertilizing the eggs of the hermaphroditic species when species with contrasting reproductive systems are involved. However, the importance of post-zygotic isolation appears to be variable according to the hybridizing partners, suggesting diverse genetic incompatibility degree and different isolation mechanisms between species (Johannesson, 2003).

7.2.4 Reproductive system and mating system

Heterozygote deficits revealed by high and significant fixation indices probably resulting from inbreeding in *F. vesiculosus* and *F. spiralis*, show a tight link between reproductive system and mating system. Indeed, hermaphroditic entities seem to present much more inbreeding than dioecious species. Also, the very low polymorphism within the two *F. spiralis* high and low entities, suggests a high selfing rate (Glémin et al., 2006). Moreover, the spatial autocorrelation analysis performed on nuclear microsatellite markers and on the psbD-rpl19 chloroplastic marker in *F. spiralis* low (the only taxon for which, this marker was polymorphic) supports the hypothesis of a very low dispersal capacity (at least for the eggs).

7.2.5 Neutrality of microsatellite markers used?

This study questions the evolution of markers and particularly the supposed neutrality of our microsatellites. Indeed, the observed pattern of differentiation between the two entities of *F. spiralis* shows a strong similarity between French and Portuguese populations occupying the same position on the shore whereas population distance from few meters but found at different heights have fixed different alleles for two loci. The use of a large number of markers like AFLPs, would permit to test if these two loci are under selection either directly or by hitchhiking (Beaumont, 2005; Nielsen et al., 2006).

7.3 Population Biology Approaches

7.3.1 Différences of resource allocation to male and female function between hermaphroditic and dioecious individuals

As expected from resource allocation theory to male and female functions (Charnov, 1982), a very small sperm/ egg ratio is observed in the hermaphroditic species *F. spiralis*. This supports the genetic analyses suggesting high inbreeding in this species, whereas reallocation towards male function is observed in the dioecious species, *F. vesiculosus* (Article 3, Billard et al, 2005b).

7.3.2 Phenological discrepancy and hybridization

The phenological discrepancy between the different individuals distributed on the shore was estimated in order to test if it could limit hybridization between *Fucus* species (Wallace effect, Ollerton, 2005). No discrepancy was shown between hermaphroditic and dioecious individuals, only a trend for a more important synchronicity of female maturity was observed. This synchronization results in the modification of the apparent sex-ratio (calculated for mature individuals). Therefore, it could favour, and not limit, hybridization via the fertilization of eggs from hermaphrodites by sperm from males. The only phenological discrepancy that was observed seemed to occur along the shore: females of the upper part were mature earlier than females of the lower part of the intertidal area. This phenomenon could limit gene flow between the lower and upper part of the shore and result at long-term in a genetic isolation. Moreover, the earliness of the females living in the upper part of the shore could be explained as a means to avoid the desiccation stress during the warmest months. Indeed, the evolution of a drought avoidance character has been shown in *Arabidopsis*

thaliana (McKay et al., 2003). Nevertheless, our results, obtained on one site and one year still need a confirmation as to their generality.

7.4 Hybridization / selection in *Fucus*

The genus *Fucus* offers a unique opportunity to study speciation and hybridization processes. Indeed, these species seem to be at different steps, more or complete, in their process of speciation, at least concerning *F. vesiculosus* and the *F. spiralis* complex. These species, living on rocky shore, are subject to different selective pressures according to their situation on the shore (Johannesson, 2003). On the lower part of the shore, strong biotic selective pressures seem to act and have induced the evolution of the reproductive system toward dioecy in *F. vesiculosus*, resulting in obligate out-crossing and the limitation of the inbreeding depression. On the contrary, in the upper part of the shore, selfing would have been favoured, limiting recombination (Takebayashi & Morrell, 2001) and maintaining the association of advantageous alleles, adapted to desiccation stress. In the case of *F. serratus*, the speciation process is more completed and it is therefore more difficult to identify the reproductive isolation mechanisms. However, it seems that strong selection acts against hybrids, suggesting in this case a genetic incompatibility between parental species (Servedio & Noor, 2003). Finally, in the case of the *F. spiralis* complex, the selection and adaptation to desiccation mechanisms seem clearly involved in the differentiation of the two groups, highlighting their interest in the study of desiccation tolerance genes.

7.5 Evolution of reproductive systems

Schiel & Foster (2006) have formulated serious doubts concerning the relevance of comparing brown algae and higher plants, principally because of our lack of knowledge about the role of microscopic stages in these species in the processes of recruitment, dispersion and colonization. However, we have noticed, that concerning the Fucaceae (which lack a developed microscopic haploid stage) theoretical expectations about the evolution of reproductive systems can be verified as well at the macro-evolutionary scale, with the evolution of dioecy from hermaphroditism (Charlesworth, 1999), as at the micro-evolutionary scale, with a reallocation of resources to sexual functions according to the mating system (Charnov, 1982). The production and dispersion of gametes seem to play a very important

role in the processes of hybridization and speciation. On one hand, the difference of resource allocation may explain the orientation of the observed hybridization; it is to say the sperm of the dioecious species fertilizing the eggs of the hermaphroditic one. On the other hand, the low dispersal capacity of these gametes can explain the limited occurrence of hybridization. Once settled by diverging selection leading to speciation, the different reproductive systems could contribute to reproductive isolation reinforcement between newly separated species (Dobzhanky, 1937; Wallace, 1889).

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APPENDICES

9 APPENDICES

APPENDIX I

Engel C. R., Billard E., Voisin M. & Viard F. (in press) Conservation and polymorphism of mitochondrial intergenic sequences in brown algae, *European Journal of Phycology*

APPENDIX II

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APPENDIX III

- Amplification protocols of microsatellite loci
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APPENDIX IV

Allelic frequencies from populations sampled along the transects

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APPENDIX I

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Conservation and polymorphism of mitochondrial intergenic sequences in brown algae

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Due to their haploid nature, uniparental inheritance and apparent absence of recombination, organellar DNA markers are ideal tools for studying both intraspecific and interspecific phylogenies. Although 'universal' cytoplasmic DNA primers have become available for both red and green seaweeds, they are not transferable to brown seaweeds. Thus, based on the sequence of mitochondrial genome of the kelp *Laminaria digitata*, we developed universal-type primers for seven intergenic spacer loci and one mononucleotide tandem repeat locus. We investigated the conservation and sequence variation of these loci among nine Laminarialean and three fucoid species, as well as intraspecific polymorphism within five of these species. Six loci were conserved in at least four of the five brown algal families tested and five were polymorphic within at least one species. Although overall levels of divergence were relatively homogenous ($\pi = 0.314 \pm 0.031$), a hierarchical analysis suggests there is variation in the rate of evolutionary change among the six intergenic spacers. Due to their high degree of transferability, versatility and to non-coding nature, we argue that these intergenic spacer loci are highly efficient markers for within-family phylogenetic studies and for intra-specific phylogeographic studies in brown seaweeds.

Key words: mitochondrial genome, PCR-based markers, intergenic spacer, marker conservation, Phaeophyceae, phylogeography

Introduction

Due to their haploid nature, uniparental inheritance and hypothetical absence of recombination, organellar DNA markers are widely used for phylogeographic and lower-level phylogenetic studies in vascular plants and animals (e.g. Avise, 2000). Such studies have been greatly facilitated by the development of 'universal' PCR-based cytoplasmic markers (e.g. plants, Taberlet *et al.*, 1991; Duminil *et al.*, 2002; animals Kocher *et al.*, 1989; Folmer *et al.*, 1994). In brown algae, particularly poorly resolved evolutionary relationships due to recent, rapid 'crown' radiation of the Phaeophyceae (de Reviers & Rousseau, 1999; Draisma *et al.*, 2001) and low intraspecific resolution of the plastid-coded RuBisCO intergenic spacer (e.g. Stache-Crain *et al.*, 1997; Yoon & Boo, 1999; Kraan & Guiry, 2000; Kraan *et al.*, 2001) have probably contributed to the relative paucity of phylogeographic studies in this group, particularly compared with green and red seaweeds (e.g. Wattier *et al.*, 2001; Wattier & Maggs, 2001; Gabrielson *et al.*, 2002; Zuccarello & West, 2002; Meusnier *et al.*, 2004; Provan *et al.*, 2005; Zuccarello *et al.*, 2005). Although 'universal' mitochondrial and plastid DNA primers have become available for both red and green algae (see Zuccarello *et al.*, 1999; Provan *et al.*, 2004), they are not transferable to brown algae, as the three major algal groups constitute separate evolutionary lineages and different endosymbiotic pathways (Bhattacharya *et al.*, 2003).

In the Phaeophyceae, the existing repertoire of mitochondrial markers has generally been limited to conserved coding regions used for higher-order phylogenetics (Ehara *et al.*, 1999; Lane *et al.*, 2006; Oudot-Le Secq *et al.*, 2006), detection of inter-specific hybridization (Coyer *et al.*, 2002*a*,*b*) and, more recently, for phylogeographic studies (Muraoka & Saitoh, 2005; Uwai *et al.*, 2006*a*). However, for lower-level taxonomic and population-level studies, presumably neutral, non-coding regions, such as introns or intergenic spacers are preferable, to avoid the confounding effects of selection, which are particularly problematic in phylogeographic studies where patterns of gene flow must be inferred from gene genealogies. Furthermore, these regions are more likely to be variable (Gielly & Taberlet, 1994), making them

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more efficient markers. For instance, intergenic spacer regions of chloroplast DNA have proved to be extremely important tools in the phylogenetic analysis of vascular plant groups, particularly at lower taxonomic levels (Kelchner, 2000). To date, of the five complete brown algal mitochondrial genomes (Oudot-Le Secq *et al*., 2002, 2006), only one possesses introns: the Ectocarpalean *Pylaiella littoralis*. On the other hand, although the brown algal mitochondrial genomes are generally very compact, intergenic spacers make up 3–7% of total genome sequence (Oudot-Le Secq *et al*., 2002, 2006). However, these spacer regions have been under-exploited. They have only been used in four studies: for taxon identification in *Ectocarpus* (Peters *et al*., 2004), intrageneric phylogeny of *Fucus* (Coyer *et al*., 2006), and for phylogeographic analysis and genetic diversity of *F. serratus* in Europe (Hoarau *et al*., 2007) and of the invasive alga, *Undaria pinnatifida* (Voisin *et al*., 2005, Uwai *et al*., 2006*b*).

By comparing two published mitochondrial genome sequences (Oudot-Le Secq *et al*., 2002), we aimed to develop efficient, 'universal' PCR-based mitochondrial markers and to assess their utility for phylogeographic studies and/or low-level phylogenies on a wide spectrum of brown seaweeds. Here, we test eight loci (seven intergenic spacer regions and one mononucleotide repeat within a coding region) on twelve ecologically important brown algal species. This suite of species covering family-, species- and population-levels was used to evaluate conservation across taxonomic levels, as well as to assess the level and utility of marker polymorphisms.

Materials and methods

Primer design

Based on the completely sequenced mitochondrial genome of *Laminaria digitata* (AJ344328; Oudot-Le Secq *et al*., 2002), we identified eight regions to test conservation and polymorphism across species (Table 1). Seven loci correspond to the longest intergenic spacer regions identified in the *L. digitata* mitochondrial genome (Oudot-Le Secq *et al*., 2002). The eighth locus, an imperfect 38-bp A/T mononucleotide simple sequence repeat region (located in a coding region), was selected because chloroplast-encoded simple sequence repeats are known for their polymorphism in higher plants (e.g. pines, Vendramin *et al*., 1996). Based on the (conserved coding) consensus sequences of *L. digitata* and *Pylaiella littoralis* (complete genome, AJ277126; Oudot-Le Secq *et al*., 2001) using Primer3 software (Rozen & Skaletsky, 2000), primers were designed in the coding sequence flanking the regions of interest, with the *L. digitata* sequence as a reference.

Samples

The conservation of candidate sequences in brown seaweeds was tested on a suite of 12 species from five, currently recognized, families in three different orders (Table 2). To assess roughly and rapidly the potential degree of nucleotide variability at the species level, two specimens of each species (from as distant as possible sites) were sequenced (Table 2). Similarly, polymorphism of the intergenic spacer loci was evaluated at the within-population level for one species from each family (i.e. *Ld*, *Up*, *Mp*, *Sp* and *Fv*), for which 16 individuals from two populations (eight individuals each) were sequenced. Only one population was available for *Sp*.

Sequencing

DNA was extracted from 5–10 mg of dried tissue using DNeasy™ Plant Mini kit (QIAGEN). Sequences were carried out on PCR products. PCR was performed in 20 μL containing 0.2 μg/μL bovine serum albumin, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01% Tween®20, 2.0 mM $MgCl_2$, 0.25 μM of each forward and reverse primer, 200 μM of each dNTP, 0.5 U Thermoprime Plus *Taq* polymerase (ABgene) and 5 μL of 1:100 diluted DNA. PCRs were run on a PTC200 thermocycler (MJ Research). After an initial denaturation step (95°C, 5 min), 'touchdown' PCR was carried out for 5 cycles of 30 s at 95°C, 30 s at 60°C, reduced by 1°C per cycle for 5 cycles, and 30 s at 72°C, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final 8 min elongation at 72°C. Recalcitrant amplifications were repeated using the same protocol, but with $MgCl_2$ concentration increased to 3.0 mM. Purified PCR products (Millipore Multiscreen-PCR plates) were sequenced in both directions using the amplification primers, purified and sequenced on an ABI 3100 capillary sequencer (Perkin-Elmer Applied Biosystems) using the BigDye kit (Perkin-Elmer Applied Biosystems), following the manufacturer's protocol.

Sequence analysis

Sequences were aligned using ClustalW (Thompson *et al*., 1994) as implemented in BioEdit 6.0.6 (Hall, 1999). With the exception of the *rps*3/*rps*19 locus, only intergenic sequences were retained for analyses; any co-amplified flanking coding sequence was thus truncated. Estimates of nucleotide diversity (π) were calculated for each locus using Arlequin 3.0 (Excoffier *et al*., 2005). To compute diversity indices, insertion-deletions (gaps) were treated as independent (mutation) sites. For comparison, we also analysed two cytoplasmic loci, for which sequences from more than three of the panel species (or closely related, congeneric species according to Yoon *et al*., 2001) were available in GenBank. These were a mitochondrial coding region locus, *nad*6, and a chloroplastic intergenic region locus, the RuBisCO spacer. Finally, for within-population analyses, mean genetic (haplotypic) diversity (H_e) was estimated across populations using Arlequin 3.0.

To assess the distribution of sequence divergence across different taxonomic levels, we carried out a

Table 1. Loci and oligonucleotide primer sequences used for tests of conservation and polymorphism in brown seaweeds.

Locus	Primer sequences	Type of sequence	Nature of sequence	Size expected (bp)/[range of size observed (bp)]	GenBank accession nos
trnP/rnl	5′-GAGGTGACGCAGTGGTAGC-3′ 5′-CGCCTATATTTTTCTTCCAAGG-3′	Intergenic spacer	Non-coding	206/[180–781]	DQ841647-DQ841668
trnK/trnA	5′-AGGGTCTTGGGTTCAATTC-3′ 5′-CACACACTCTACCAACTGAGTTAT-3′	Intergenic spacer	Non-coding	176	NA
trnW/trnI	5′-GGGGTTCAAATCCCTCTCTT-3′ 5′-CCTACATTGTTAGCTTCATGAGAA-3′	Intergenic spacer	Non-coding	292/[257–299]	DQ841669-DQ841692
rps3/rps19	5′-AAAATAAAATAAAATCGTGGTTCG-3′ 5′-TTAAACCCGAAATGGTTGG-3′	Mononucleotide (A/T) simple sequence repeat	Coding	196/[174–195]	DQ841589-DQ841606
ORF384/*atp*6	5′-AGGTATTTACCGTGAAAGTGC-3′ 5′-CAACACCGAAACTTAAAAGAGG-3′	Intergenic spacer	Non-coding	398/[383–398]	DQ841575-DQ841578
rps14/atp8[a]	5′-GCGCAAAGCGTGTTGGTAA-3′ 5′-CGCTAAAGAAGGTAATATGAAACG-3′	Intergenic spacer	Non-coding	342/[279–337]	DQ841579-DQ841588
atp8/trnS[a]	5′-TGTACGTTTCATATTACCTTCTTTAGC-3′5′-TAGCAAACCAAGGCTTTCAAC-3′	Intergenic spacer	Non-coding	244/[182–245]	DQ841607-DQ841624
rpl31/rns	5′-CCAGTGTGGACAGGAAAACG-3′ 5′-CTCTGAGCCAGGATCAAACTC-3′	Intergenic spacer	Non-coding	346/[272–350]	DQ841625-DQ841646

Abbreviation: NA: no sequence generated.
Notes: Locus names correspond to flanking coding regions based on the *L. digitata* mitochondrial genome and are presented in the order of appearance after the origin of replication (Oudot-Le Secq et al., 2002). Expected size based on the *L. digitata* mitochondrial genome.
[a]When the complete mitochondrial genome of *Fucus vesiculosus* was published, after sequencing for this study had been completed, degenerate primers were designed and found to amplify the three *Fucus* species and *L. digitata* using the same PCR conditions (see Material and Methods): *atp*8/*trnS*, forward primer 5′-TKTACGTTTYATRYTRCCTTCTTTAGC-3′, reverse primer 5′-TAGCAAACCAAGGCTTTCAYC-3′; *rps*14/*atp*8, forward primer 5′-KCGCAAAKCKTGTTGGTAA-3′, reverse primer 5′-MGCTAAAGAAGGYARYATRAAACG-3′.

three-level hierarchical analysis of molecular variance (AMOVA, Excoffier *et al*., 1992). The total variance in the number of mutations between molecular haplotypes was partitioned into (co)variance components due to differences among families/orders, among species within families/orders and within species. The RuBisCO spacer was also included in this analysis, as three to 12 sequences were available for five of the panel species (GenBank accession numbers given in footnote of Table 3). For trnW/trnI, which showed the highest level of conservation, a Minimum-Evolution tree was constructed using MEGA version 3.1 (Kumar *et al*., 2004). Nodal support was estimated with 1000 re-samplings (bootstraps) on the dataset.

Results

Conservation and divergence across species

All eight loci not only amplified in *L. digitata* but also in at least one other species (Table 3A), validating our primer design. GenBank accession numbers for sequences obtained across species are given in Table 1. Locus *trn*K/*trn*A produced two-banded profiles for all specimens except *Sp* and the Fucaceae. Since the aim of this study was to provide PCR-based markers that do not necessitate time-consuming cloning procedures, this locus was discarded from further analysis. Of the seven remaining loci, conservation was variable, with two to 12 species amplifying out of the 12 tested species. The highest rate of conservation was observed for *trn*W/*trn*I and the lowest for ORF384/*atp*6, which amplified only in *Ld* and *Lh*. In addition, the rate of cross-amplification decreased with increasing taxonomic levels: the highest rates of amplification were found in the Laminariales (five to seven loci), followed by the Tilopteridalean *Sp* (four loci) and finally the Fucales (the three Fucaceae species) (three loci; Table 3B). For *trn*W/*trn*I, which was conserved in every species, a Minimum-Evolution tree revealed phylogenetic relationships in agreement with published literature (Fig. 1). For example, *Sp* was not included in the Laminariales (*sensu stricto*) clade (Sazaki *et al*., 2001). Loci generally showed taxonomically coherent amplification patterns, as those that amplified in the Fucaceae also amplified in the Laminariales, with the exception of *Mp* at the *rpl*31/*rns* locus. Only *rps*14/*atp*8 showed a variable amplification pattern with at least one non-amplifying species in each of the three families within the Laminariales.

All sequences could be aligned attesting to their probable homology. In general, there were as many haplotypes as there were species that amplified (compare n_{sp} and n_h in Table 3A) and some haplotype sharing was detected among the different *Fucus* species at *trn*P/*rnl* and *trn*W/*trn*I (data not shown). At the interspecific level polymorphism was high and an average of 70%

Table 2. List of species used in this study and their collection locations.

Order	Family	Species	Taxon label	Site	Coordinates	Collector
Laminariales	Laminariaceae	*Laminaria digitata* (Hudson) J.V. Lamouroux	Ld1	Saint Aubin, Jersey	49°10′N; 2°11′W	C. Billot
(*sensu stricto*)			Ld2	Cape Gris-Nez, France	50°52′N; 1°35′E	C. Billot
			Population 1 ($n=8$)	Audresselles, France	50°49′N; 1°36′E	C. Billot
			Population 2 ($n=8$)	Veyran Bay, UK	50°13′N; 4°49′E	C. Billot
		L. hyperborea (Gunnerus) Foslie	Lh1	Plouguerneau, France	48°36′N; 04°33′W	C. Destombe
			Lh2	Bergen, Norvège	60°25′N; 4°57′E	M. Skage
		L. ochroleuca Bachelot de la Pylaie	Lo1	Viana, Portugal	41°41′N; 08°51′W	M. Valero, C. Daguin
			Lo2	Aber Wrac′h, France	48°36′N; 04°34′W	L. Lévêque, F. Viard
		L. saccharina (Linnaeus) J.V. Lamouroux[a]	Ls1	Roscoff, France	48°44′N; 03°59′W	C. Engel
			Ls2	Brest Marina, France	48°23′N; 04°26′W	M. Valero, Co. Destombe, C. Engel
	Alariaceae	*Alaria esculenta* (Linnaeus) Greville	Ae1	Plouguerneau, France	48°36′N; 04°33′W	C. Destombe
			Ae2	Schoodic Point, Maine	44°20′N; 68°03′W	M. Coleman
		Undaria pinnatifida (Harvey) Suringar	Up1	St Malo, France	48°38′N; 02°00′W	C. Engel, F. Viard
			Up2	Santa Barbara, California	34°25′N; 119°42′W	B. Kinlan
			Population 1 ($n=8$)	St Malo, France	48°38′N; 02°00′W	C. Engel, F. Viard
			Population 2 ($n=8$)	Otago Harbor, New Zealand	45°53′S; 170°28′E	C. Hurd
	Lessoniaceae	*Macrocystis pyrifera*[b](Linnaeus) Agardh	Mp1	Ancud, Chile	41°51′S; 73°50′W	S. Faugeron, F. Viard
			Mp2	Niebla, Chile	39°51′S; 73°23′W	S. Faugeron, F. Viard

			Population 1 ($n=8$)	Ancud, Chile	41°51′S; 73°50′W	S. Faugeron, F. Viard
			Population 2 ($n=8$)	Niebla, Chile	39°51′S; 73°23′W	S. Faugeron, F. Viard
		Lessonia nigrescens Bory de Saint-Vincent	Ln1	Las Cruces, Chile	33°30′S; 71°37′W	S. Faugeron, E. Martinez, F. Viard
			Ln2	Caleta San Marco, Chile	20°48′S; 70°12′W	S. Faugeron, E. Martinez, F. Viard
Tilopteridales[c]	Phyllariaceae	*Saccorhiza polyschides* (Lightfoot) Batters	Sp1	Plouguerneau, France	48°36′N; 04°33′W	C. Destombe
			Sp2	Roscoff, France	48°44′N; 03°59′W	C. Destombe, C. Engel
			Population 1 ($n=8$)	Roscoff, France	48°44′N; 03°59′W	C. Engel, D. Gautier
Fucales	Fucaceae	*Fucus vesiculosus* Linnaeus	Fv1	Cape Gris-Nez, France	50°52′N; 1°35′E	C. Engel, C. Daguin
			Fv2	Viana, Portugal	41°41′N; 08°51′W	C. Engel, C. Daguin
			Population 1 ($n=8$)	Cape Gris-Nez, France	50°52′N; 1°35′E	C. Engel, C. Daguin
			Population 2 ($n=8$)	Viana, Portugal	41°41′N; 08°51′W	C. Engel, C. Daguin
		F. spirali linnaeus	Fs1	Cape Gris-Nez, France	50°52‘N; 1°35′E	C. Engel, C. Daguin
			Fs2	Viana, Portugal	41°41′N; 08°51′W	C. Engel, C. Daguin, E. Serrao, M.. Valero
		F. ceranoides Linnaeus	Fc1	St. Laurent, France	47°53′N; 3°57′W	C. Daguin
			Fc2	Penzé, France	48°38′N; 3°57′W	C. Daguin

[a]Based on molecular evidence, Lane et al. (2006) recently proposed a new taxonomic position for this species and proposed the name *Saccharina latissima*; [b]see Yoon et al. (2001) and Lane et al. (2006) for proposed changes in the taxonomic status of *Macrocystis,* in particular, Lane et al. (2006) moved *Macrocystis* to the Laminariaceae family; [c]proposed by Sasaki et al. (2001).

Table 3. Conservation and polymorphism of loci across the 12 study species.

	Intergenic spacers							Coding sequences	
Locus	trn W/trnI	rpl 31/rns	trn P/rnl[a]	atp 8/trnS	rps 14/atp8	ORF 384/atp6	RuBisCo spacer[b]	rps 3/rps19	nad6[c]
A. Synthesis of results for conservation and polymorphism tests at the among-species level									
n_{sp}	12	11	11 (10)	9	5	2	9	9	8
n_{seq}	24	22	22 (20)	18	10	4	9	18	8
n_h	14	18	11 (10)	13	7	2	9	14	8
length (bp)	243	259	662 (87)	128	213	338	286	195	947
S (bp)	179	222	635 (56)	124	146	123	212	65	496
S_{indels} (bp)	111	132	601 (71)	90	75	66	126	28	166
S_{sub} (bp)	132	173	57 (26)	85	94	57	126	46	386
π	0.319	0.395	0.195 (0.333)	0.391	0.343	0.243	0.369	0.106	0.197
B. Detailed patterns of conservation and tests of within-species polymorphism (S (bp); $n_{seq} = 2$, except when otherwise stated)									
Ld	0	0	0	1	0	0	0 ($n_{seq} = 3$; $n_h = 1$)	0	4($n_{seq} = 2$; $n_h = 2$)
Lh	0	1	0	0	1	0	ND[d]	0	ND
Lo	0	0	0	0	0	–	ND	0	ND
Ls	0	1	0	1	–	–	NA	2	NA
Ae	1	4	0	0	–	–	2($n_{seq} = 7$; $n_h = 3$)	4	NA
Up	24	7	0	1	2	–	5($n_{seq} = 12$; $n_h = 4$)	0	NA
Ln	16	10	1	42	–	–	NA	5	NA
Mp	0	–	0	0	0	–	NA	3	ND
Sp	0	2	–	0	–	–	0($n_{seq} = 4$; $n_h = 1$)	7	ND
Fv	0	0	1	–	–	–	2($n_{seq} = 3$; $n_h = 2$)	–	NA
Fs	0	1	0	–	–	–	NA	–	ND
Fc	1	0	0	–	–	–	ND	–	ND

Abbreviations: n_{sp}: number of species amplifying at the locus; n_{seq}: number of sequences analysed; n_h: number of haplotypes detected; length: aligned sequence length; S: total number of polymorphic sites, including indels (note that some sites show both substitutions and indels); S_{indels}: number of observed sites with insertion-deletions; S_{sub}: number of observed sites with substitutions; π: nucleotide diversity; –: no amplification; NA: irrelevant, only one sequence available; ND: not done or no sequence available. Species abbreviations are given in the text.
Notes: [a]Data in parentheses correspond to analysis done without *Lh* in the panel. [b]Sequences used: *L. digitata* (AF318971, AF318972[d], AY851559), *L. saccharina* (AF318980), *A. esculenta* (AF109795–AF109800, AF318958), *U. pinnatifida* (DQ133188-DQ133196, AF109805, AY851535), *M. pyrifera* (AF318998), *L. nigrescens* (AY851544), *S. polyschides* (AB045253-AB045256), *F. vesiculosus* (AF346700, AF132474, AY878074), *F. spiralis* (AY246553). [c]Sequences used: *L. digitata* (AY857921, AJ344328), *L. sinclairii* (AY857920), *L. sessile* (AY857928), *A. esculenta* (AY878857), *U. pinnatifida* (AY857912), *L. nigrescens* (AY857929), *M. integrifolia* (AY857915), *F. vesiculosus* (AY494079). [d]Accession listed as *Lh* but proved to be *Ld* (see Erting et al., 2004).

(standard error [SE], 10%) of the sites were variable (Table 3A). Of these polymorphic sites, insertion–deletions (indels or gaps) constituted a large proportion of the variable sites, ranging from 43% (*rps*3/*rps*19) to 95% (*trn*P/*rnl*; Table 3A), and the indels were generally blocks of sequence, with median sizes of *ca.* 5 to 20 bp. In particular, at the *trn*P/*rnl* locus, *Lh* showed a long insertion of 575 bp, lacking from all other species. Omitting this species from the panel reduced the indel proportion at the *trn*P/*rnl* locus to 46%. Variability of the *rps*3/*rps*19 locus was not confined to the poly (A/T) region. Furthermore, both synonymous (17) and non synonymous (30) substitutions were found in this coding sequence.

Sequence divergence was generally high, except at the *rps*3/*rps*19 locus, which showed the lowest π value of the seven tested loci. However, this locus, chosen for its A/T repeat region (Table 1), only contains coding sequence. Its level of divergence is similar to that observed in the *nad*6 locus, also only containing coding sequence (Table 3A). All six intergenic spacer loci showed comparable π values (Table 3A), particularly if *Lh*, and thus a 575 bp indel, is omitted from the *trn*P/*rnl* locus' panel ($n_{sp} = 10$, $\pi = 0.333$). In addition, divergence of these mitochondrial intergenic spacers was of the same order as that found using the chloroplastic RuBisCO spacer (Table 3A). Interestingly, nucleotide diversity across loci was not correlated with the number of species amplified (Spearman rank coefficient, $r_S = 0.109$, $P = 0.82$). For example, the *rps*14/*atp*8 locus, amplified in only five Laminarialean species, showed comparable π values to those loci that amplified in 11 or more species (Table 3A).

Polymorphism at lower taxonomic levels (species and populations)

Although based on two individuals per species, except for *Lo* all species showed at least one polymorphic site in at least one locus (Table 3B). *Ln* showed the highest intraspecific polymorphism with an average of 14.8 (SE, 7.2) polymorphic sites per locus. Some loci revealed more intraspecific variability than others: *rps*3/*rps*19 and *rpl*31/*rns*

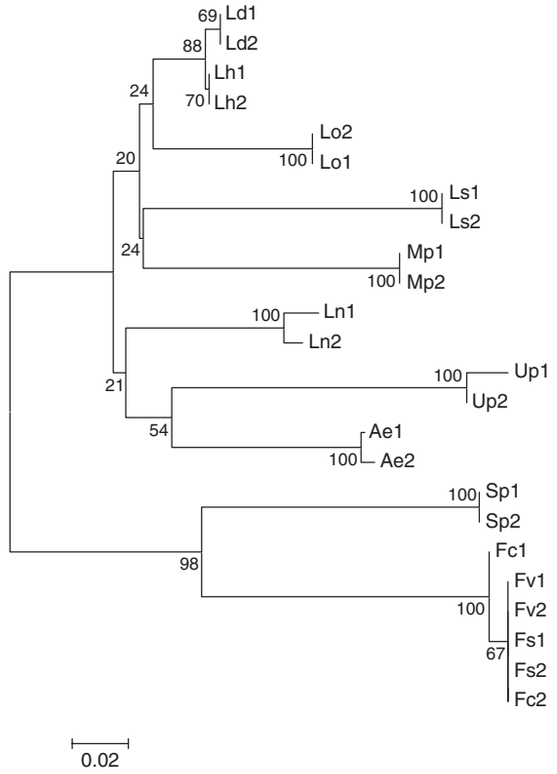

Fig. 1. *trn*W/*trn*I tree based on a Minimum-Evolution reconstruction method using Kimura's 2-parameter distance. Nodal support was estimated with 1000 re-samplings of the dataset (bootstraps). Taxon labels correspond to those given in Table 2.

both showed polymorphism within five or more species, while ORF384/*atp*6, *trn*P/*rnl* and *rps*14/*atp*8 showed no or very low variability (Table 3B).

At the within-population level, polymorphism was detected at each tested locus for at least one species (Table 4), with two to three haplotypes detected within at least one population.

Distribution of sequence divergence across taxonomic levels

Whatever the locus, inter-specific variability was always higher than intra-specific variability, demonstrating the usefulness of these loci for the study of species-level phylogenetic relationships. However, excluding ORF384/*atp*6, which showed no intra-specific variability and was not conserved beyond the family level, the hierarchical analysis of sequence divergence revealed two types of pattern (Fig. 2). The three loci (*trn*P/*rnl*, *rps*14/*atp*8 and *rpl*31/*rns*) (group 1) that showed the lowest intra-specific divergence had a quasi-linear progression of sequence divergence; most variation was found at the among-family level followed by the within-family level. For the other three loci (group 2), the within-family component accounted for as much, or more, sequence variability than the among-family component. Therefore the rate of molecular evolution varied between the different loci, demonstrating that, in spite of their physical linkage, different markers are appropriate for studies at different taxonomic levels. The first group, whose pattern was shared by the RuBisCO spacer (Fig. 2), is suitable for phylogenetic studies at the family level, for which the chloroplast marker is frequently employed (e.g. Yoon *et al.*, 2001). The faster rates of evolution of the second group provide markers that are more appropriate for lower taxonomic levels, e.g. at the intrafamilial or generic levels, and even for intra-specific, phylogeographic studies. Nevertheless, the moderate inter-specific variability of *rpl*31/*rns* (group 1) and the high rate of intraspecific variability (Table 3B) suggest that it could be useful across several taxonomic levels. These patterns were independent of the numbers of species that amplified.

Discussion

In this study, we found seven loci that were conserved to various degrees among five brown algal families, from three orders. The high degree of conservation of these loci demonstrated their potential utility for intrafamilial gene genealogy studies and for intraspecific phylogeographic studies. Due to the non-coding nature of the intergenic spacers developed here, the faster mutation rates make for highly efficient markers, providing relatively short sequences with a substantial amount of polymorphism for less sequencing effort.

In accordance with the proposed rapid 'crown' radiation of the Phaeophyceae, (e.g. Draisma *et al.*, 2001; Rousseau *et al.*, 2001), six of the seven loci worked in at least four families, demonstrating high conservation of the chosen loci. This high degree of transferability is congruent with the apparent conservation of gene order across mitochondrial genomes of brown seaweeds (Oudot-Le Secq *et al.*, 2006), thereby facilitating the development of 'universal' primers, as in this study.

The experimental design of this study was not appropriate for phylogenetic analyses, however, the ME tree constructed with the *trn*W/*trn*I dataset (Fig. 1) was concordant with current phylogenies of kelp species, suggesting that this set of markers could be useful for phylogeny reconstruction. Although more work is needed on the mitogenomics of the Laminariales, the conservation of loci across species appears to be phylogenetically fairly informative. Indeed, most loci were conserved within a family and within the

Table 4. (A) Gene diversity (H_e) and (B) number of haplotypes at the within-species level for each intergenic locus.

Locus	trnW/trnI	rpl31/rns	trnP/rnl	atp8/trnS	rps14/atp8	ORF384/atp6
A. Gene diversity H_e averaged over populations						
Ld ($n_{pop}=2$)	0.000	0.232	0.000	0.125	0.000	0.125
Up ($n_{pop}=2$)	0.423	0.339	0.000	0.423	0.000	–
Mp ($n_{pop}=2$)	0.000	–	0.000	0.000	0.143	–
Sp ($n_{pop}=1$)	0.000	0.000	–	ND	–	–
Fv ($n_{pop}=2$)	0.000	ND	0.214	–	–	–
B. Number of haplotypes						
Ld Pop 1/Pop 2	1/1	1/3	1/1	2/1	1/1	2/1
Up Pop 1/Pop 2	2/3	1/2	1/1	2/3	1/1	–
Mp Pop 1/Pop 2	1/1	–	1/1	1/1	1/2	–
Sp Pop 1	1	1	–	ND	–	–
Fv Pop 1/Pop 2	1/1	ND	2/1	–	–	–

Abbreviations: n_{pop}: number of populations analysed; –: no sequence available (see Table 2); ND: not done.

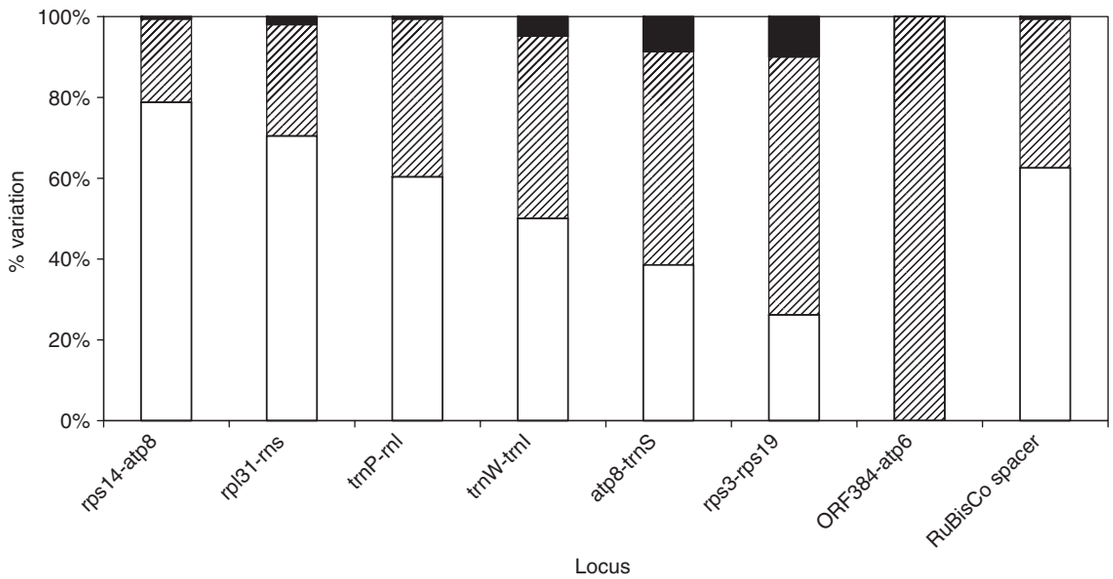

Fig. 2. Distribution of sequence divergence across three taxonomic levels in the seven tested loci. Variance components for locus *trn*P/*rnl* are presented without *Lh* in which a unique 575-bp insertion masked the pattern of variation. White bars: among-family level; hatched bars: among-species/within-family level; black bars: within-species level.

Laminariales. The few exceptions were generally concordant with the current phylogenies (Yoon *et al*., 2001; Lane *et al*., 2006). For example, two loci (*rps*14/*atp*8 and *rpl*31/*rns*) amplify in only one of the two Lessoniaceae species of the tested taxa, *Ln* and *Mp*, but both Yoon *et al*., (2001) and Lane *et al*., (2006) demonstrated that these are paraphyletic.

Although a comparison of mtDNA gene maps (*cf*. Oudot-Le Secq *et al*., 2006) shows that all *L. digitata* loci identified for primer design in this study are present in *F. vesiculosus*, three did not amplify in any Fucaceaen species. A lower rate of transferability to the Fucaceae is in accordance with the phylogenetic relationships among the Phaeophyceae (Draisma *et al*., 2001; Rousseau *et al*., 2001), which places the Fucales as an earlier divergent group compared with the Laminariales (*sensu stricto*). Differences in the conserved coding genes flanking the intergenic spacers (or mononucleotide repeat region) may have resulted in poor hybridization of these primers in the Fucaceae. Since sequencing was completed for the present study, the complete *F. vesiculosus* mtDNA sequence has been published (GenBank accession no. AY494079, Oudot-Le Secq *et al*., 2006). Checking our primer sequences against the published sequence revealed four to seven substitutions for at least one primer in each of three loci that did not amplify in the Fucaceae (i.e. *rps*3/*rps*19, *rps*14/*atp*8 and *atp*8/*trn*S; results not shown). Consequently, designing new primers allowing for degenerate sites may increase the cross-amplification success of the markers developed here. We tested this hypothesis by designing degenerate primers

(see footnote in Table 1) for two intergenic loci that failed to amplify in *Fucus* species (*atp*8/*trn*S and *rps*14/*atp*8**).** Using the same PCR conditions, the loci were successfully amplified with these degenerate primers in *Fucus* and *L. digitata* (used as a positive control).

As expected and as seen in chloroplast markers of higher plants (e.g. Gielly & Taberlet, 1994), our mitochondrial intergenic spacers are very efficient and clearly evolve faster than previously used mitochondrial coding sequences of brown seaweeds (Ehara *et al*., 1999; Kogame *et al*., 2005; Muraoka & Saitoh, 2005; Coyer *et al*., 2006; Lane *et al*., 2006; Uwai *et al*., 2006). Indeed, even within single loci, flanking coding sequences showed divergence values 2- to 12-fold lower than their corresponding non-coding spacer regions (results not shown). These coding flanking sequences also showed much lower values of nucleotide diversity (π values of 0.027 to 0.061) than that observed in the coding sequence *rps*3/*rps*19, except for the flanking coding sequence in *rps*31/*rns*, which was comparable ($\pi = 0.167$). However, none of our sequences contain the entire gene-coding region, precluding any conclusions on molecular evolutionary mechanisms or the demonstration of selective constraints acting on the observed polymorphism.

At the population level, based on only 16 individuals from two populations, all five tested species showed at least one polymorphic locus out of the six, except for *Sp* (where only one population was tested). Mean gene diversities ranged from 0.125 to 0.423 (Table 4A), adequate levels of polymorphism for comprehensive phylogeographic studies. Nucleotide diversity values varied for similar levels of gene diversities (data not shown), indicating that intra-specific variability may arise from indels as well as from simple base pair substitutions (see also Voisin *et al.* 2005). Haplotypes were generally shared between the two study populations (with the exception of the *trn*W-*trn*I locus in *Up*) and up to three variants were observed within a single population ($n = 8$; Table 4B). In accordance with our hierarchical analysis, both *trn*W/*trn*I and *atp*8/*trn*S show the highest levels of variability (see *Up*, Table 4B). However, no clear patterns of locus-specific polymorphism emerged from this preliminary survey, suggesting that different loci may be useful in different species. In addition, species-specific patterns of population structure and/or historical demographic events will also affect intra-population polymorphism. For example, using *atp*8/*trn*S, a phylogeographic study of *Ln* along the Chilean coast showed a highly structured pattern of haplotype distribution, in accordance with the observed high intra-specific polymorphism detected, based on only two (distantly sampled) individuals (Table 3B; Valero M, personal communication).

Compared with other available cytoplasmic markers, these intergenic spacer loci have proved to be highly efficient markers at the intraspecific level. For example, at the population level, the intraspecific sequence divergence revealed in *Undaria pinnatifida* ($\pi = 0.0139$, 333 bp) using concatenated *atp*8/*trn*S and *trn*W/*trn*I loci (Voisin *et al*., 2005) was of the same order as that reported for interspecific divergence using the RuBisCO spacer in all three *Undaria* species ($\pi = 0.008$, 268 bp, Yoon & Boo, 1999).

The six intergenic spacer loci tested here come from different parts of the mitochondrial genome (see Fig. 1 in Oudot-Le Secq *et al*., 2002). Although the overall levels of divergence are relatively homogenous among the six intergenic spacers (π values in Table 3A), our hierarchical analysis suggests there is some variation in the rates of evolutionary change among the different mtDNA regions (Fig. 2). Therefore, different markers are probably appropriate for investigations at different taxonomic levels, although the usefulness of a particular locus may vary between groups that are assumed to occupy the same evolutionary level (see Kelchner, 2000). In addition to the mutation rate and historical events, polymorphism is affected by generation time. Nevertheless, since these spacer loci are relatively short, one or more loci can be combined and concatenated for analysis, depending on the degree of variability required (e.g. see Voisin *et al*., 2005). Alternatively, close loci may be combined into a larger sequence region including both coding and non-coding regions, as in a recent study of the phylogenetics of *Fucus* (Coyer *et al*., 2006). In conclusion, the mitochondrial loci described here should prove to be useful tools for evolutionary and genetic studies at lower taxonomical levels, including the infraspecific level, in brown algae.

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APPENDIX II

ANNEX 2

Exemples of Sequence Alignment of the rubisco-spacer (in bold) and part of Rbc Large Sub-Unit and Small Sub-Unit. When sequences were monomorphic within species and/or within lineage, one sequence is shown

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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                             10         20         30         40         50         60         70
F.vesiculosus 1     TTTGGTGGTG GTACAATCGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCGAATCGT GTTGCGTTAG
F.vesiculosus 2     TTTGGTGGTG GTACAATCGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCGAATCGT GTTGCGTTAG
F.ceranoides        TTTGGTGGTG GTACAATCGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCGAATCGT GTTGCGTTAG
F.spiralis          TTTGGTGGTG GTACAATCGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCGAATCGT GTTGCGTTAG
F.evanescens 1      TTTGGTGGTG GTACAATCGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCGAATCGT GTTGCGTTAG
F.evanescens 2      TTTGGTGGTG GTACAATCGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCGAATCGT GTTGCGTTAG
F.serratus          TTTGGTGGTG GTACAATCGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCGAATCGT GTTGCGTTAG
P.canaliculata      TTTGGTGGTG GTACAATTGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCGAATCGT GTAGCGCTAG
A.nodosum           TTTGGTGGTG GTACAATTGG TCACCCTGAT GGGATACAAG CAGGTGCTAC AGCGAATCGT GTTGCGTTAG
S.compressa         TTTGGTGGTG GTACAATTGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCGAATCGT GTTGCGTTAG
H.harveyanus        TTTGGTGGTG GTACAATCGG CCACCCTGAT GGTATACAAG CAGGTGCTAC AGCGAATCGT GTTGCATTAG
P.limitata          TTTGGTGGTG GTACAATCGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCGAATCGT GTTGCGTTAG
X.chondrophylla     TTTGGTGGTG GTACAATTGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCAAACCGT GTTGCTTTAG
X.chondrophylla     TTTGGTGGTG GTACAATTGG TCACCCTGAT GGTATACAAG CGGGTGCTAC AGCAAACCGT GTTGCTTTAG
X.gladiata          TTTGGTGGTG GTACAATTGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCAAACCGT GTTGCTTTAG
H.banskii           TTTGGTGGTG GTACAATTGG TCACCCTGAT GGTATACAAG CAGGTGCTAC AGCAAACCGT GTAGCATTAG


                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                             80         90        100        110        120        130        140
F.vesiculosus 1     AAGCTATGGT TTTAGCTCGT AATGAAGGTC GTGATTATGT TGGTGAAGGT CCTGAAATTT TACGTACAGC
F.vesiculosus 2     AAGCTATGGT TTTAGCTCGT AATGAAGGTC GTGATTATGT TGGTGAAGGT CCTGAAATTT TACGTACAGC
F.ceranoides        AAGCTATGGT TTTAGCTCGT AATGAAGGTC GTGATTATGT TGGTGAAGGT CCTGAAATTT TACGTACAGC
F.spiralis          AAGCTATGGT TTTAGCTCGT AATGAAGGTC GTGATTATGT TGGTGAAGGT CCTGAAATTT TACGTACAGC
F.evanescens 1      AAGCTATGGT TTTAGCTCGT AATGAAGGTC GTGATTATGT TGGTGAAGGT CCTGAGATTT TACGTACAGC
F.evanescens 2      AAGCTATGGT TTTAGCTCGT AATGAAGGTC GTGATTATGT TGGTGAAGGT CCTGAGATTT TACGTACAGC
F.serratus          AAGCTATGGT TTTAGCTCGT AATGAAGGTC GTGATTATGT TGGTGAAGGN CCTGAGATTT TACGTACAGC
P.canaliculata      AAGCTATGGT TTTAGCTCGT AATGAAGGGC GTGATTATGT GGGTGAAGGC CCTGAGATTT TACGTACAGC
A.nodosum           AAGCTATCGT TTTAGCTCGT AATGAGGGTC GTGATTATGT TGGTGAAGGT CCTGAGATTT TACGTACAGC
S.compressa         AAGCTATGGT TTTAGCTCGT AATGAGGGTC GTGATTATGT TGGTGAAGGT CTTGAGATTT TACGTACAGC
H.harveyanus        AAGCTATGGT TTTAGCTCGT AATGAAGGTC GTGATTATGT TGGTGAAGGT CCTGAGATTT TACGTACAGC
P.limitata          AAGCTATGGT TTTAGCTCGT AATGAAGGTC GTGATTATGT TGGTGAAGGT CCTGAGATTT TACGTACAGC
X.chondrophylla     AAGCTATGGT TTTAGCTCGT AATGAAAGTC GAGACTACGT AGGTGAAGGT CCTGAAATTT TACGTACAGC
X.chondrophylla     AAGCCATGGT TTTAGCTCGT AATGAAGGTC GAGACTACGT AGGTGAAGGT CCTGAAATTT TACGTACAGC
X.gladiata          AAGCTGTGGT TTTAGCTCGT AATGAAGGTC GAGACTATGT AGGTGAAGGT CCTGAAATTT TACGTACAGC
H.banskii           AGGCTATGGT TTTAGCTCGT AATGAAGGTC GTGACTATGT TGGTGAAGGT CCTGAGATTT TACGTACAGC


                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            150        160        170        180        190        200        210
F.vesiculosus 1     TGCTAGTACT TGTGGACCAT TAAAAGCAGC TTTAGATTTA TGGAAAGATA TTACTTTTGA ATATACTTCA
F.vesiculosus 2     TGCTAGTACT TGTGGACCAT TAAAAGCAGC TTTAGATTTA TGGAAAGATA TTACTTTTGA ATATACTTCA
F.ceranoides        TGCTAGTACT TGTGGACCAT TAAAAGCAGC TTTAGATTTA TGGAAAGATA TTACTTTTGA ATATACTTCA
F.spiralis          TGCTAGTACT TGTGGACCAT TAAAAGCAGC TTTAGATTTA TGGAAAGATA TTACTTTTGA ATATACTTCA
F.evanescens 1      TGGTAGTACT TGTGGGCCAT TAAAAGCAGC TTTAGATTTA TGGAAAGATA TTACTTTTGA ATATACTTCA
F.evanescens 2      TGGTAGTACT TGTGGGCCAT TAAAAGCAGC TTTAGATTTA TGGAAAGATA TTACTTTTGA ATATACTTCA
F.serratus          TGGTAGTACT TGTGGACCAT TAAAAGCAGC TTTAGATTTA TGGAAAGATA TTACTTTTGA ATATACTTCA
P.canaliculata      TGGTGGTACT TGTGGACCAT TAAAAGCAGC TTTAGATTTA TGGAAAGATA TTACTTTTGA ATATACTTCA
A.nodosum           TGGTAGTACT TGTGGACCAT TAAAAGCAGC TTTAGATTTA TGGAAAGATA TTACTTTTGA ATATACTTCA
S.compressa         TGGTAATACT TGTGGACCAT TAAAAGCAGC TTTAGATTTA TGGAAAAATA TTACTTTTGA ATATACTTCA
H.harveyanus        CGGTAGTACT TGTGGACCAT TAAAAGCAGC TTTAGATTTA TGGAAAAATA TTACTTTTGA ATATACTTCA
P.limitata          TGGTAGTACT TGTGGCCCAT TAAAAGCAGC TTTAGATTTA TGGAAAGATA TTACTTTTGA ATATACTTCA
X.chondrophylla     TGCAAGTACT TGTGGACCAT TAAAAGCCGC TTTAGATCTA TGGAAAGATA TTACTTTTGA GTATACTTCA
X.chondrophylla     TGCAAGTACT TGTGGACCAT TAAAAGCAGC TTTAGATCTA TGGAAAGATA TTACTTTTGA GTATACTTCA
X.gladiata          TGCAAGTACT TGTGGTCCAT TAAAAGCAGC TTTAGATCTA TGGAAAGATA TTACCTTTGA GTATACTTCA
H.banskii           TGCTAGTACT TGTGGACCAT TAAAAGCAGC TTTAGATTTA TGGAAAGATA TTACTTTTGA TTATACTTCA
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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            220        230        240        250        260        270        280
F.vesiculosus 1     ACAGATACAC CTGATTTCAC TGAAGTGGCA ACTGAAAGTA ACTAAATAAT A-TATTAAAA GTAGTATA--
F.vesiculosus 2     ACAGATACAC CTGATTTCAC TGAAGTGGCA ACTGAAAGTA ACTAAATAAT A-TATTAAAA GTAGTATA--
F.ceranoides        ACAGATACAC CTGATTTCAC TGAAGTGGCA ACTGAAAGTA ACTAAATAAT A-TATTAAAA GTAGTATA--
F.spiralis          ACAGATACAC CTGATTTCAC TGAAGTGGCA ACTGAAAGTA ACTAAATAAT A-TATTAAAA GTAGTATA--
F.evanescens 1      ACAGATACAC CTGATTTCGT TGAAGTGGCA ACTGAAAGTA ACTAAATAAT A-TATTAAAA GTAGTATA--
F.evanescens 2      ACAGATACAC CTGATTTCGT TGAAGTGGCA ACTGAAAGTA ACTAAATAAT A-TATTAAAA GTAGTATA--
F.serratus          ACAGATACAC CTGATTTCGT TGAAGTGGCA ACTGAAAGTA ACTAAATAAT A-TATTAAAA TTA-------
P.canaliculata      ACAGATACAC CTGATTTCGT TGAAATGGCA ACTGAAAGTA ACTAAATAAT A-TATTAAAA GTAGTATA--
A.nodosum           ACAGATACAC CTGATTTCAC TGAAGTGGCA ACTGAAAGTA ACTAAATAAT A-TATAGTAA GTATTATA--
S.compressa         ACAGATACAC CTGATTTCGT TGAAGTGGCA ACTGAAAGTA ACTAAATAAT A-TATAGTAA ATATCATA--
H.harveyanus        ACAGATACAC CTGATTTCGT TGAAGTGGCA ACTGAAAGTA ACTAAATAAT A-TATTAAAA TTAGTATA--
P.limitata          ACAGATACAC CTGATTTCGT TGAAGTGGCA ACTGAAAGTA ACTAAATAAT A--------- ----------
X.chondrophylla     ACAGATACAC CTGATTTTGT TGAAGTTGAA ACTGGAAGTA ACTAAT---- -----CACGA ATATTATATT
X.chondrophylla     ACAGATACAC CTGATTATGT TGAAGTTGAA ACTGAAAGTT AAGTAT---- --TTTTACGA GTATTATATT
X.gladiata          ACAGATACAC CTGATTTTGT TGAAGTTGAA ACTGGAAGTA ACTAATAAGT ATTATCATGA ATATTATATT
H.banskii           ACAGATACAC CTGATTTCGT TGAAGTTGCT ACTGAAAGTA AATAAATATT A-TATTGAAA GTA-------

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            290        300        310        320        330        340        350
F.vesiculosus 1     ATGTTTAC-- ----TATAAA GAAGATCAAA GTTTAAGTTT AAATAAGATT TTCTTTATAG TAAA-ATTCT
F.vesiculosus 2     ATGTTTAC-- ----TATAAA GAAGATCAAA GTTTAAGTTT AAATAAGATT TTCTTTATAG TAAA-ATTCT
F.ceranoides        ATGTTTAC-- ----TATAAA GAAGATCAAA GTTTAAGTTT AAATAAGATT TTCTTTATAG TAAA-ATTCT
F.spiralis          ATGTTTAC-- ----TATAAA GAAGATCAAA GTTTAAGTTT AAATAAGATT TTCTTTATAG TAAA-ATTCT
F.evanescens 1      ATGTTTAC-- ----TATAAA GAAGATCAAA GTTTA----- -AATAAGATT TTCTTTATAG TAAA-ATTCT
F.evanescens 2      ATGTTTAC-- ----TATAAA GAAGATCAAA GTTTA----- -AATAAGATT TTCTTTATAG TAAA-ATTCT
F.serratus          ---------- ---------- ---------- ---------- -AATAAGATT TTCTTTATAG TAAA-ATTCT
P.canaliculata      ATACTTAC-- ----TATAAA GAGGATCAAA GTTTAAGCTT AAATTAGGTT TTCTTTATAG TAAAAAGT-T
A.nodosum           ATATTTAA-- ----TATAAG GAAGATCAAA GTTCAAGTTT AAATTAAGTT TTCTTTATAG TAAAAAAT-T
S.compressa         ATATTTAC-- ----TATAAA GAAGATCAAA ATTTAAGTTT AAATTAAATT TTTTTT-TAT TAAGAATT-T
H.harveyanus        ATGCTTAG-- ----TATAAA GAAGATC--- ---------- ---------- TTCTTTATAC TAAGCATTAT
P.limitata          ---------- ---------- ---------- ---------- ---------- ---------- ---------T
X.chondrophylla     ACATTCAC-G ATAATATAAA GAAGAGTAAA GTTTAATTTT AAATTTGATT TTCTTTATAG TAAAAAGT-T
X.chondrophylla     ATATTCACAG GTAATATAAA GAAGATTAAA GTTTAATTTT AAATTTGATT TTCTTTATAG TAAAAAGT-T
X.gladiata          ACATTCAC-G ATAATATAAA GAAGAGTAGA GTTTAATTTT AAATTTGATT TTCTTTCTAG TAAAAAGT-T
H.banskii           ---------- ---------- ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            360        370        380        390        400        410        420
F.vesiculosus 1     ATTAATTTTT TAAAAATTTT TAATACTTTA CATTAAAGTT AAATAAATAA ATGGTTTAGT AGTTAACTAA
F.vesiculosus 2     ATTAATTTTT TAAAAATTTT TAATACTTTA CATTAAAGTT AAATAAATAA ATGGTTTAGT AGTTAACTAA
F.ceranoides        ATTAATTTTT TAAAAATTTT TAATACTTTA CATTAAAGTT AAATAAATAA ATGGTTTAGT AGTTAACTAA
F.spiralis          ATTAATTTTT TAAAAATTTT TAATACTTTA CATTAAAGTT AAATAAATAA ATGGTTTAGT AGTTAACTAA
F.evanescens 1      ATTGATTTTT AAAAAATTTT TAATACTTTA CATTAAAGTT AAATAAATAA ACAGTTTAGT AGTTAACTAA
F.evanescens 2      ATTAATTTTT AAAAAATTTT TAATACTTTA CATTAAAGTT AAATAAATAA ACAGTTTAGT AGTTAACTAA
F.serratus          ATTAATTTTT AAAAAATTTT TAANACTTTA CATTAAAGTT AAATAAATAA ACAGTTTAGT AGTTAACTAA
P.canaliculata      ATTGTTATTT TAAAAATTTT TAATACTTTA CATTAAAGTT AAATAAATAA AAAATTTAGT AGTTAACTAA
A.nodosum           ATTAGTATTT TATAAATTTT TAATACTTTA CATTAAAGTT AAATAAATAA AAAGTTTAGT AGTTAACTAA
S.compressa         ATTAGTATTT TGAAAATTTT TAATACTTTA CATTAAAGTT AAATAAATAA AAAGTTTAGT AGTTAACTAA
H.harveyanus        ACTAATTTTA AAAAAATTTT TAATACTTTA CATTAAAGTT AAATAAATAA AAAGTTTAGT AGTTAACTAA
P.limitata          ACTAAGTTTA AAAAAATTTT TAATACTTTA CATTAAAGGT AAATAAATAA AAAGTTTAGT AGTTAACTAA
X.chondrophylla     A--------- --AAAATTTT TAGTACTTTA TATTAAAGTT AAATATATAA AAAGTTTAGT AGTTAACTAA
X.chondrophylla     A--------- --AAAATTTT TAATACTTTA TATTAAAGTT AAATAGATAG AAAGTTTAGT AGTTAACTAA
X.gladiata          A--------- --AAAATTTT TAATACTTTA TATTAAAGTT AAATAGATAA AAAGTTTAGT AGTTAACTAA
H.banskii           ---------- --ATAATTTT TAATATTTTA TTTTAAAGTG AAATAGATAA AATTTTTAGT AGTTAACTAA
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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            430        440        450        460        470        480        490
F.vesiculosus 1     AAACAAAAAT TTCAGATTTA TATTAAATAA CATATTTGAA GAGTGATGAG ACTTACACAA GGATGTTTTT
F.vesiculosus 2     AAACAAAAAT TTCAGATTTA TATTAAATAA CATATTTGAA GAGTGATGAG ACTTACACAA GGATGTTTTT
F.ceranoides        AAACAAAAAT TTCAGATTTA TATTAAATAA CATATTTGAA GAGTGATGAG ACTTACACAA GGATGTTTTT
F.spiralis          AAACAAAAAT TTCAGATTTA TATTAAATAA AATATTTGAA GAGTGATGAG ACTTACACAA GGATGTTTTT
F.evanescens 1      AAACAAAAAT TTCAGATTTA TATTAAATAA AATATTTGAA GAGTGATGAG ACTTACACAA GGATGTTTTT
F.evanescens 2      AAACAAAAAT TTCAGATTTA TATTAAATAA AATATTTGAA GAGTGATGAG ACTTACACAA GGATGTTTTT
F.serratus          AAACAAAAAT TTCANATTTA TATTAAATAA AATATTTGAA GAGTGATGAG ACTTACACAA GGATGTTTTT
P.canaliculata      AAACAAAAAT TTCAGATTTA TATTAAATAA AATATTTGAA GAGTGATGAG ACTTACACAA GGATGTTTTT
A.nodosum           AAGCAAAAAT TTCATATTTA TATTAAATAA AATATTTGAA GAGTGATGAG ACTTACACAA GGATGTTTTT
S.compressa         AAGCAAAAAT TTCATATTTA TATTAAATAA AATATTTGAA GAGTGATGAG ACTTACACAA GGATGTTTTT
H.harveyanus        AAACAAAAAT TTCAGATTTA TATTAAATAA AATATTTGAA GAGTGATGAG ACTTACACAA GGATGTTTTT
P.limitata          AAACAAAAAT TTCATATTTA TATTAAATAA AATATTTGAA GAGTGATGAG ACTTACACAA GGATGTTTTT
X.chondrophylla     AAACAAAATT TTCAGATTTA TATTGAATAA AATATTTGAA GAGTGATGAG ACTTACACAA GGGTGTTTTT
X.chondrophylla     AAACAAAATT TTCATACTTA T------TAA AATATTTGAA GAGTCATGAG ACTTACACAA GGGTGTTTTT
X.gladiata          AAACAAAATT TTCAGATTTA TATTGAATAA AATATTTGAA GAGTCATGAG ACTTACACAA GGGTGTTTTT
H.banskii           AAATAAAATT TTTAGATTTA TATTGAATAA AATATTTGAA AAATAATGAG ACTTACACAA GGATGTTTTT


                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..
                            500        510        520        530        540        550
F.vesiculosus 1     CATTTTTACC AGATTTAAGT GATGAGCAAA TTAAAATC-- AATTAATTAT GCTATTTCAA AA
F.vesiculosus 2     CATTTTTACC AGATTTAAGT GATGAGCAAA TTAAAAATCA AATTAATTAT GCTATTTCAA AA
F.ceranoides        CATTTTTACC AGATTTAAGT GATGAGCAAA TTAAAAATCA AATTAATTAT GCTATTTCAA AA
F.spiralis          CATTTTTACC AGATTTAAGT GATGAGCAAA TTAAAAATCA AATTAATTAT GCTATTTCAA AA
F.evanescens 1      CATTTTTACC AGATTTAAGT GATGAGCAAA TTAAAAATCA AATTAATTAT GCTATTTCAA AA
F.evanescens 2      CATTTTTACC AGATTTAAGT GATGAGCAAA TTAAAAATCA AATTAATTAT GCTATTTCAA AA
F.serratus          CATTTTTACC AGATTTAAGT GATGAGCAAA TTAAAAGTCA CATTAATTAT GCTATTTCAA AA
P.canaliculata      CATTTTTACC AGATTTAAGT GATGAGCAAA TTAAAAGCCA AATTAATTAT GCTATTTCAA AA
A.nodosum           CATTTTTACC AGATTTAAGT GATGAGCAAA TTAAAAATCA AATTAATTAT GCTATTTCAA AA
S.compressa         CATTTTTACC AGATTTAAGT GATGAACAAA TTAAAAATCA AATTAATTAT GCTATTTCAA AA
H.harveyanus        CATTTTTACC AGATTTAAGT GATGAGCAAA TTAAAAGTCA AATTAATTAT GCTATTTCAA AA
P.limitata          CATTTTTACC AGATTTAAGT GATGAGCAAA TTAAAAGTCA AATTAATTAT GCTATTTCAA AA
X.chondrophylla     CATTTTTACC AGATTTAAAT GATGAGCAAA TTAAAAATCA AATTAATTAT GCTATTTCAA AA
X.chondrophylla     CATTTTTACC AGATTTAAAC GATGAGCAAA TTAAAAATCA AATTAATTAT GCTATTTCAA AA
X.gladiata          CATTTTTACC AGATTTAAAT GATGAGCAAA TTAAAAATCA AATTAATTAT GCTATTTCAA AA
H.banskii           CATTTTTACC AGATTTAAGT GATGAGCAAA TTAAAAATCA AATTGATTAT GCTATTTCAA AG
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Alignment: thiG-ycf54

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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                             10         20         30         40         50         60         70
F.vesiculosus 1     TGAAACGTT- ---------- ---------- ---------- -------ATT TTTAGAAGTA GTTTGGCAAT
F.vesiculosus 2     TGAAACGTT- ---------- ---------- ---------- -------ATT TTTAGAAGTA GTTTGGCAAT
F.spiralis          TGAAACGTT- ---------- ---------- ---------- -------ATT TTTAGAAGTA GTTTGGCAAT
F.ceranoides 1      TGAAACGTT- ---------- ---------- ---------- -------ATT TTTAGAAGTA GTTTGGCAAT
F.ceranoides 2      TGAAACGTT- ---------- ---------- ---------- -------ATT TTTAGAAGTA GTTTGGCAAT
F.serratus          TGAAACGTT- ---------- ---------- ---------- -------ATT TTTAGAAGTA GTTTGTCAAT
H.harveyanus        TGAAACGTT- ---------- ---------- ---------- -------ATT TTTAGAAGTA GTTTGGCAAT
P.limitata          TGAAACGTT- ---------- ---------- ---------- -------ATT TTTAGAAGTA GTTTGGCAAT
P.canaliculata      TGAAACGTT- ---------- ---------- ---------- -------ATT ATTAGAAGTA GTTTGGTAAT
A.nodosum           TGAAACGTT- ---------- ---------- ---------- -------ATT TTTAGAAGTA GTTTAGTAAT
S.compressa         TGAAACGTT- ---------- ---------- ---------- -------ATT TTTAGAAGTA GTTTGGTAAT
S.babingtonii       TGAAAGATTA AGATTTTGCT GGTTTTTAAC TGAGGGTATA CCCGAAGAGT TTAAGAAGTA GTTTGGTAAT
H.banskii           TAAAA----- ---------- ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                              80         90        100        110        120        130        140
F.vesiculosus 1     TTTTTTT-TA ATAGTCAAAA TTTTAATCAT ATATTACAAT GTATAAA--- -------TAT AAGTATAAAT
F.vesiculosus 2     TTTTTTT-TA ATAGTCAAAA TTTTAATCAT ATATTACAAT GTATAAA--- -------TAT AAGTATAAAT
F.spiralis          TTTTTTT-TA ATAGTCAAAA TTTTAATCAT ATATTACAAT GTATAAA--- -------TAT AAGTATAAAT
F.ceranoides 1      TTTTTTT-TA ATAGTCAAAA TTTTAATCAT ATATTACAAT GTATAAA--- -------TAT AAGTATAAAT
F.ceranoides 2      TTTTTTT-TA ATAGTCAAAA TTTTAATCAT ATATTACAAT GTATAAA--- ---------- ---------T
F.serratus          TTTTTTT-TA ATAGTCAAAA TTTTAATCAT ATATTACAAT GTATAAA--- -------TAT AAGCATAAAT
H.harveyanus        TTTTTTT-TA ATAGTCAAAA TGTTAATCCT ATATTACAAT GTATAAA--- -------TAT AAGCATAAAT
P.limitata          TTTTTTT-TA ATAGTCAAAA TGTTAATCAT ATATTACAAT GTATAAA--- -------TAT AAGCATAAAT
P.canaliculata      TTTTTT--TA ATAATCAAAA TGTTAATCAT ATATTACAAT GTATAATTAT ATATAAATTT AAGCATAAAC
A.nodosum           TTTTTTTTTA ACAGGCAAAA TGTTAATCAT ATATTACCAT GTATAATTAT ATATAAATAT AAGCGTAAAA
S.compressa         TTTATTA-TA ATAGGTAAAA TATTAATAAT ATATTACCAT TTATAAT--- ----AAATAT AAGTATAAAA
S.babingtonii       TTTTTT--TA ATAGGCAAAA TGTTAATCAT ATATTACCAT GTATAAT--- ----AAATAT AAGTATAAAA
H.banskii           ---------- ---------- TATTATTTTT TTGATAATAT TT-TAAT--- -----TTTTT CAATATAAAT

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                             150        160        170        180        190        200        210
F.vesiculosus 1     TACTTAATTT AAATTTTATA AATTCTTGAG AAGGATAATT AAATCTATTA A-AAAACAAT AAAAAATTAT
F.vesiculosus 2     TACTTAATTT AAATTTTATA AATTCTTGAG AAGGATAATT AAATCTATTA A-AAAATAAT AAAAAATTAT
F.spiralis          TACTTAATTT AAATTTTATA AATTCTTGAG AAGGATAATT AAATCTATTA A-AAAACAAT AAAAAATTAT
F.ceranoides 1      TACTTAATTT AAATTTTATA AATTCTTGAG AAGGATAATT AAATCTATTA A-AAAACAAT AAAAAATTAT
F.ceranoides 2      TACTTAATTT AAATTTTATA AATTCTTGAG AAGGATAATT AAATCTATTA A-AAAACAAT AAAAAATTAT
F.serratus          TACTTAATTT GAATTTTGTA AATTCTTGAG AAGGATAATT AAATCTATTA A-AAAACAAT AAAAAATTAT
H.harveyanus        TATTTAAATT GAATTTTGTA AGTTTTTGAG AATGGTAATT AAATCTATTA A-AAGACAAT AAAAAATTAT
P.limitata          TACTTAAATT GAATTTTGTA AATTCTTGAA AATGATAATT AAATCTATTA A-AA-ACAAT AAAAAATTAT
P.canaliculata      TACTTAAATT GAATTTTGTA AATTCTTAAG AATGAGAAGT AAATTTATTA A-AAAACAAC AAAAAATTAT
A.nodosum           TACTTAAATT GAATTTTGTA AATTCTTGAG AATGAGAAGT AAATCTATTA AAAAAACAAT AAAAAATTAT
S.compressa         TCCTTAAATT GAATTTTGTA AATTCTTGAG AATGAGAAGT AAATCTATTA AAAAAACAAT AAAAAATTAT
S.babingtonii       TACTTAAATT GAATTTTGTA AATTCTTGAG AATGAGAAGT AAATCTATTA AAAAAACAAT AAAAAATTAT
H.banskii           TAAATAAATT TAATTTT--- ---TCATAGA ATTATTTTGA AAA------A ACAAAATAAT AAAAGATTAT

                    ....|....| ....|....| ....|....| ....|....| ....|
                             220        230        240        250
F.vesiculosus 1     TTTCAATTTT AAAAAATAAA TAGAATATAG ATTAATTAAT AAAA-
F.vesiculosus 2     TTTCAATTTT AAAAAATAAA TAGAATATAG ATTAATTAAT AAAA-
F.spiralis          TTTCAATTTT AAAAAATAAA TAGAATATAG ATTAATTAAT AAAA-
F.ceranoides 1      TTTCAATTTT AAAAAATAAA TAGAATATAG ATTAATTAAT AAAA-
F.ceranoides 2      TTTCAATTTT AAAAAATAAA TAGAATATAG ATTAATTAAT AAAA-
F.serratus          TTTCAATTTT AAAAAATAAA TAGAATATAG ATTAATTAAT AAAA-
H.harveyanus        TTTCAATTTT AAAAAATAAA TAGAATATAG ATTAATTAAT AAAA-
P.limitata          TTTCAATTTT AAAAAATAAA TAGAATATAG ATTAATTAAA AAAAA
P.canaliculata      TTTCTATTTT AAAAAATAAA TAGAATATAT ATTAATTAAA AAAA-
A.nodosum           TTTTAATTTT TAAAAATAAA TAAAATAAAG ATTAATTAAA AAAA-
S.compressa         TTTCAATTTT TAAAAATAAA TAAAATATAG ATTAATTAAT AAAA-
S.babingtonii       TTTCAATTTT TAAAAATAAA TAAAATCTAG ATTAATTAAT AAAA-
H.banskii           TTTCAATTTT TAAAAATAAA TAAAATATAA ATTAATTAAT AAAA-
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Alignment: psbX-ycf66

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                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                           10         20         30         40         50         60         70
F.vesiculosus 1   AATTTTAAAA ATAGATTATT TATATG---- ---------- ---------- ---ATTTTA- ----TTTTTT
F.vesiculosus 2   AATTTTAAAA ATAGATTATT TATATG---- ---------- ---------- ---ATTTTA- ----TTTTTT
F.spiralis 1      AATTTTAAAA ATAGATTATT TATATG---- ---------- ---------- ---ATTTTA- ----TTTTTT
F.spiralis 2      AATTTTAAAA ATAGATTATT TATATG---- ---------- ---------- ---ATTTTA- ----TTTTTT
Fceranoides       AATTTTAAAA ATAGATTATT TATAAG---- ---------- ---------- ---ATTTTA- ----TTTTTT
F.cotonii         AATTTTAAAA ATAGATTATT TATATG---- ---------- ---------- ---ATTTTA- ----TTTTTT
F.serratus 1      AATTTTAAAA ATAGATTATT TATATGGTTT TGCTTTTTAA ATAAAATATA AATATTTTA- ----TTTTTT
F.serratus 2      AATTTTAAAA ATAGATTATT TATATGGTTT TGCTTTTTAA ATAAAATATA AATATTTTA- ----TTTTTT
A.nodosum         AATTTAAAAC ATAGATTATT TATATGATTT TACTTTTTAA ATAAAATATT TATATTATAC TTAATTTTTT
H.harveyanus      AATTTAAAAA ATAAATTATG TATATCGTTT TACTTTTTAA ATAAAATAGT TATATTTTAT TTAATTTTTT
P.limitata        ---------- ---------- ---------- ---------- ---AAATATT TATATTTTAT TT--------
P.canaliculata    AATTTAAAAA ATAGATTATT TATATAGCTT TACTTTTTAA ATAAAATATT -ATATTTTA- ----TTCTTT
S.compressa       AATTTAAAAC ATAGGTTATT TATAAGGTTT TATTTTTTAA ATAAAATATT TATATTTTAC TTAATTTTTT
S.babingtonii     AATGTAAAAC ATAGGTTATT TATATGGTTT TACTTTTTAA ATAAAATATT TATATTTT-- ----------
S.siliquosa       AATGTAAAAC ATAGGTTATT TATATGGTTT TACTTTTTAA AGAAAATATT TATATTTT-- ----------
H.banskii         AATATAAAAA ATATATTATT TCTATGGTTT TATTTTTTTA ATAAAATATA AAT------- ----------

                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                           80         90        100        110        120        130        140
F.vesiculosus 1   TATATTATTC ATGGAATAAG GTTTTAAATT TATATTTTTA ATAAAGTCTA TTAAAATTTT AGA-------
F.vesiculosus 2   TATATTATTC ATGGAATAAG GTTTTAAATT TATATTTTTA ATAAAATCTA TTAAAATTTT AGA-------
F.spiralis 1      TATATTATTC ATGGAATAAG GTTTTAAATT TATATTTTTA ATAAAATCTA TTAAAATTTT AGA-------
F.spiralis 2      TATATTATTC ATGGAATAAG GTTTTAAATT TATATTTTTA ATAAAGTCTA TTAAAATTTT AGA-------
Fceranoides       TATATTATTC ATGGAATAAG GTTTTAAATT TATATTTTTA ATAAAGTCTA TTCAAATTTT AGA-------
F.cotonii         TATATTATTC ATGGAATAAG GTTTTAAATT TATATTTTTA ATAAAATCTA TTAAAATTTT AGA-------
F.serratus 1      TATATTATTC ATGGAATAAG GTTTTAAATT TATATTTTTA ATAAAGTCTA TTCGAATTTT AGA-------
F.serratus 2      TATATTATTC ATGGAATAAG GTTTTAAATT TATATTTTTA ATAAAGTCTA TTCGAATTTT AGA-------
A.nodosum         TATATTATTT ATTGAATAAT ATTTTAAATT TATATTTTTA ATATAGTCTA TTCACATTTT AGA------A
H.harveyanus      T--------- ---------- --------TT TATTTATGAA ATAAAGTCTA TTCCAATTTT ATA------A
P.limitata        ---------- ---------- ---------- ---TTATGGA ATAAAATCTA TTCTAATTTT AAA------A
P.canaliculata    TATATTATTT ATTAAATAAG GTTTTAA--- ----TTTTTA ATAAAGTCTA TTCAAATTTT AGATTTAGAA
S.compressa       TACATCATTT ATTGAATAAT ATTTTAAATT TATATTTTTA ATAAAGTCTA TTCATGTTTT AGA------A
S.babingtonii     ---------- ---------- ---------- --------TA ATAAAGTCTA TTCATGTTTT AGA------A
S.siliquosa       ---------- ---------- ---------- --------TA ATAAAGTCTA TTCATGTTTT AA-------A
H.banskii         ---------- ---------- --------GT TATATT---- AAAAAGTATA TTTAAATTTT AGA------A

                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          150        160        170        180        190        200        210
F.vesiculosus 1   ------AAAT ATTATTATAA TACTGTCTGG ATATATTTAA ATATAT-TAA AGTTTATAGG AAATCAAGAA
F.vesiculosus 2   ------AAAT ATTATTATAA TACTGTCTGG ATATATTTAA ATATAT-TAA AGTTTATAGG AAATCAAGAA
F.spiralis 1      ------AAAT ATTATTATAA TACTGTCTGG ATATATTTAA ATATAT-TAA AGTTTATAGG AAATCAAGAA
F.spiralis 2      ------AAAT ATTATTATAA TACTGTCTGG ATATATTTAA ATATAT-TAA AGTTTATAGG AAATCAAGAA
Fceranoides       ------AAAT ATTATTATAA TACTGTCTGG ATATATTTAA ATATAT-TAA AGTTTATAGG AAATCAAGAA
F.cotonii         ------AAAT ATTATTATAA TACTGTCTGG ATATATTTAA ATATAT-TAA AGTTTATAGG AAATCAAGAA
F.serratus 1      ------AAAT ATTATTATAA TACTGTCTGG ATATATT--- --------AA AGTTTATAGG AAATCAAGAA
F.serratus 2      ------AAAT ATTATTATAA TACTGTCTGG ATATATTTAA ATATAT-TAA AGTTTATAGG AAATCAAGAA
A.nodosum         CATAAGTAAT ATTATTATAA TACTATAT-- --ATATTGAA ATATAT-TAA ATTTTATAAG AAATCAAGAA
H.harveyanus      CATAAGTAAT ATTATTATAA TACTATCTGG ---------- ATATAT-TAA AGTTTATATG AAATCCAGAA
P.limitata        CATAAGTAAT ATTATTGTAA TACTATCTAG ---------- ATACAT-TAA AGTTTATATG AAATCCGGAA
P.canaliculata    CATAAGTAAT ATTTTTAGAA CAGTATATGG AGATATTTAA ATATAT-TAA AGTTTTTAGG AAATCAAGAA
S.compressa       CATAAGTAAT ATTACTATAA TATTCTAT-- --------AG ATATAT-TAA AGTTTATAGG AAATCAAGAA
S.babingtonii     CATAAGTAAT ATTA------ -----TAT-- --------AG ATATAT-TAA AGTTTATAGG AAATCAAGAA
S.siliquosa       CATAAGTAAT ATTA------ -----TAT-- --------AG ATATAT-TAA AGTTTATAGG AAATCAAGAA
H.banskii         CATAATTAAT ATTATTATAA TAATCGAT-- -------TAT ATATATGTAA AGTTTATATA AAATCAAAAA
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                  ....|....| ....|....| ....|....| ....|....| ....|....| ..
                          220        230        240        250        260
F.vesiculosus 1   TTATTAACAT TTTAACTATC TAATAAATAA ATTTTTCAAT ATTTAAAGGT TT
F.vesiculosus 2   TTATTAACAT TTTAACTATC TAATAAATAA ATTTTTCAAT ATTTAAAGGT TT
F.spiralis 1      TTATTAACAT TTTAACTATC TAATAAATAA ATTTTTCAAT ATTTAAAGGT TT
F.spiralis 2      TTATTAACAT TTTAACTATC TAATAAATAA ATTTTTCAAT ATTTAAAGGT TT
Fceranoides       TTATTAACAT TTTAACTATC TAATAAATAA ATTTTTCAAT ATTTAAAG-- --
F.cotonii         TTATTAACAT TTTAACTATC TAATAAATAA ATTTTTCAAT ATTTAAAGGT TT
F.serratus 1      TTATTAACAT TTTAACTATC TAATAAATAA ATTTTTCAAT ATTTAAAGGT TT
F.serratus 2      TTATTAACAT TTTAACTATC TAATAAATAA ATTTTTCAAT ATTTAAAGGT TT
A.nodosum         GTATTAACAT TTTAGTTATC TAATAAATAA ATTTTCCAAT ATTTAAAGGT TT
H.harveyanus      TTATTAATAT TTTAAACATT TAATAAATAA ATTTTCCAAT ATTTAAAGGT TT
P.limitata        TTATTAACAT TTTAACCATT TAATAAATAA ATTTTCTAAT ATTTAAAGGT TT
P.canaliculata    TTATTAACAT TTTAACCATC TAATAAAAAA ATTTTCCAAT ATTTAAAGGT TT
S.compressa       TTATTAACAT TTTAATTATC TAATAAATAA ATTTTCCAAT ATTTAAAGGT TT
S.babingtonii     TTATTAACAT TTTAATTATC TAATAAATAA ATTTTCCAAT ATTTAAAGGT TT
S.siliquosa       TTATTAACAT TTTAATTATC TAATAAATAA ATTTTCCAAT ATTTAAAGGT TT
H.banskii         TTATTAAAAT TTTAACTATT TAATAAATAA ATTTTCTAAT ATTCAAAAAT TT
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APPENDIX III

APPENDIX III

PCR conditions for amplification of each microsatellite locus

PCR MIX for 20µl (4µl of 1/250 diluted DNA)		
reagent	concentration	volume
H2O		4.7 µl
Bovin Serum Albumin (BSA)	1 mg/ml	4.0 µl
Buffer	10X	2.0 µl
MgCl2	25 mM	1.6 µl
dNTPs	2.5mM	1.6 µl
Unlabelled Forward Primer *	5µM	1.0 µl
Unlabelled Reverse Primer*	5µM	0.7 µl
Labelled Reverse Primer*	5µM	0.3 µl
Taq	5U/µl	1.0 µl

- *Whether forward or reverse primer were labelled

PCR conditions for amplification of psbD-rpl19 locus

PCR MIX for 20µl (4µl of 1/250 diluted DNA)		
reagent	concentration	volume
H2O		4.7 µl
Bovin Serum Albumin (BSA)	1 mg/ml	4.0 µl
Buffer	10X	2.0 µl
MgCl2	25 mM	1.6 µl
dNTPs	2.5mM	1.6 µl
Unlabelled Forward Primer *	5µM	0.8 µl
Unlabelled Reverse Primer*	5µM	0.8 µl
Labelled Forward Primer*	5µM	0.2 µl
Labelled Reverse Primer *	5µM	0.2 µl
Taq	5U/µl	1.0 µl

PCR conditions for amplification of Rbc-spacer, thiG-ycf54 and psbX-ycf66 loci

PCR MIX for 20µl (4µl of 1/250 diluted DNA)		
reagent	concentration	volume
H2O		4.3 µl
Bovin Serum Albumin (BSA)	1 mg/ml	4.0 µl
Buffer	10X	2.0 µl
MgCl2	25 mM	2.0 µl
dNTPs	2.5mM	1.6 µl
Forward Primer	5µM	10. µl
Reverse Primer	5µM	1.0 µl
Taq	5U/µl	1.0 µl

Restriction conditions for Rbc-spacer and psbDrpl19 loci

Restriction MIX for 10µl (5µl of PCR mix)		
reagent	concentration	volume
H2O		3.5 µl
Buffer	10X	1.0 µl
Enzyme SspI	5000 U/ml	0.5 µl
Enzyme AseI	10000 U/ml	0.5 µl

Restriction sites of the enzymes SspI, cuting in the rubisco-spacer sequence and of AseI, cutting between the two single nucleotide repeats in the intergenic region psbD-rpl19

Enzyme	SspI	AseI
Restriction site	5'…AAT²ATT…3' 3'…TTA²TAA…5'	5'…AT²TAAT…3' 3'…TAAT²TA…5'

APPENDIX IV

ANNEX 4: allelic frequencies locus by locus of clusters sampled along transects in Portugal and France

L20	Populations :	vesP6	vesP5	vesF6	vesF5	spiLP6	spiLP5	spiLF6	spiLF5	spiHP6	spiHP5	spiHF6	serF6	serF5	hybP6	hybP5	hvspF6	HvspF5	hvseF6	HsevF5	HsespF5
Number of individuals		47	77	89	30	34	27	48	51	16	16	19	26	67	8	27	9	7	8	4	2
Number of Alleles		7	8	14	9	3	1	1	6	1	1	1	7	11	3	7	7	4	7	4	3
Frequency	120	0	0.019	0	0.018	0.956	1	1	0.94	0	0	0	0	0,067	0.625	0.389	0.389	0.714	0	0	0.5
Frequency	123	0	0	0	0	0	0	0	0	0	0	0	0.019	0	0	0	0	0	0	0	0
Frequency	129	0	0	0.006	0	0	0	0	0.02	0	0	0	0	0,030	0	0	0	0.071	0	0	0
Frequency	132	0.063	0.026	0.006	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Frequency	135	0	0	0.028	0	0	0	0	0	0	0	0	0.269	0,246	0	0.056	0.056	0	0.063	0.167	0.25
Frequency	138	0	0	0.107	0.232	0	0	0	0	0	0	0	0.173	0,112	0	0	0.056	0.071	0.063	0	0
Frequency	141	0.219	0.019	0.219	0.054	0.015	0	0	0.01	0	0	0	0.365	0,313	0.063	0	0.056	0.143	0.5	0.333	0.25
Frequency	144	0.016	0	0.202	0.214	0	0	0	0	0	0	0	0.096	0,082	0	0	0.167	0	0.063	0	0
Frequency	147	0.641	0.779	0.202	0.036	0.029	0	0	0	1	1	1	0.058	0,052	0.313	0.463	0.056	0	0.125	0	0
Frequency	150	0.016	0.13	0.202	0.071	0	0	0	0	0	0	0	0	0,007	0	0.019	0	0	0	0	0
Frequency	153	0	0	0.202	0.25	0	0	0	0.01	0	0	0	0.019	0,052	0	0.019	0.222	0	0.125	0.167	0
Frequency	156	0	0	0.202	0.107	0	0	0	0	0	0	0	0	0,030	0	0	0	0	0.063	0	0
Frequency	159	0.016	0	0.202	0	0	0	0	0	0	0	0	0	0,007	0	0.037	0	0	0	0.333	0
Frequency	162	0.031	0	0.202	0	0	0	0	0.01	0	0	0	0	0	0	0	0	0	0	0	0
Frequency	165	0	0.006	0.202	0.018	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Frequency	168	0	0.013	0.202	0	0	0	0	0.01	0	0	0	0	0	0	0.019	0	0	0	0	0
Frequency	174	0	0.006	0.202	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Proportion of heterozygotes		0.219	0.247	0.202	0.679	0.029	0	0	0.12	0	0	0	0.769	0.810	0.125	0.259	0.667	0.286	0.75	0.667	0.5
Gene Diversity (Nei)		0.545	0.377	0.202	0.831	0.086	0	0	0.117	0	0	0	0.765	0.817	0.542	0.641	0.804	0.495	0.75	0.867	0.833

vesP6: *F. vesiculosus* transect Portugal 2006
vesP5: *F. vesiculosus* transect Portugal 2005
vesF6: *F. vesiculosus* transect France 2006
vesF5: *F. vesiculosus* transect France 2005
spiLP6: *F. spiralis* Low transect Portugal 2006
spiLP5: *F. spiralis* Low transect Portugal 2005
spiLF6: *F. spiralis* Low transect France 2006
spiLF5: *F. spiralis* High transect France 2005
spiHP6: *F. spiralis* High *s* transect Portugal 2005
spiHP5: *F. spiralis* High transect Portugal 2005
spiHF6: *F. spiralis* High transect France 2006

serF6: *F. serratus* transect France 2006
serF5: *F. serratus* transect France 2005* no amplification for fsp1 and fsp2

hybP6: hybrids *F.vesiculosus/F. spiralis* Portugal 2006
hybP5: hybrids *F.vesiculosus/F. spiralis* Portugal 2005
hvspF6 : hybrids *F.vesiculosus/F. spiralis* France 2006
hvspF5 : hybrids *F.vesiculosus/F. spiralis* France 2005
hvseF6 : hybrids *F.vesiculosus/F. spiralis* France 2006
hvseF5 : hybrids *F.vesiculosus/F. spiralis* France 2005
hsespF5 : hybrids Fserratus/Fspiralis France 2005

L38	Populations :	vesP6	vesP5	vesF6	vesF5	spiLP6	spiLP5	spiLF6	spiLF5	spiHP6	spiHP5	spiHF6	serF6	serF5	hybP6	hybP5	hvspF6	HvspF5	hvseF6	HsevF5	HsespF5
Number of individuals		47	77	89	30	34	27	48	51	16	16	19	26	67	8	27	9	7	8	4	2
Number of Alleles		4	4	13	9	1	1	1	2	1	1	1	10	15	3	4	4	2	10	4	1
Frequency	160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.063	0	0
Frequency	163	0	0	0.017	0.017	0	0	0	0	0	0	0	0	0	0	0	0	0	0.063	0	0
Frequency	166	0	0	0.017	0.017	0	0	0	0	0	0	0	0	0	0	0	0	0	0.125	0	0
Frequency	169	0.628	0.481	0.32	0.417	1	1	1	0.98	1	1	1	0	0	0.75	0.796	0.667	0.929	0.125	0.25	1
Frequency	172	0	0	0.09	0.033	0	0	0	0	0	0	0	0	0	0	0	0.056	0	0	0	0
Frequency	181	0.011	0.078	0.034	0.017	0	0	0	0	0	0	0	0	0	0	0.019	0	0	0.063	0	0
Frequency	190	0.34	0.325	0.315	0.333	0	0	0	0.02	0	0	0	0	0,023	0.125	0.148	0.222	0.071	0.188	0.25	0
Frequency	193	0	0	0.045	0.117	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0.063	0.25	0
Frequency	196	0	0	0.062	0.017	0	0	0	0	0	0	0	0.038	0,083	0	0	0.056	0	0.063	0	0
Frequency	199	0	0	0.062	0.033	0	0	0	0	0	0	0	0.115	0,106	0	0	0	0	0.125	0	0
Frequency	202	0	0	0	0	0	0	0	0	0	0	0	0.115	0,106	0	0	0	0	0	0	0
Frequency	205	0	0	0.011	0	0	0	0	0	0	0	0	0.231	0,151	0	0	0	0	0.125	0.25	0
Frequency	208	0.021	0.117	0.017	0	0	0	0	0	0	0	0	0.269	0,197	0.125	0.037	0	0	0	0	0
Frequency	211	0	0	0	0	0	0	0	0	0	0	0	0.154	0,151	0	0	0	0	0	0	0
Frequency	214	0	0	0	0	0	0	0	0	0	0	0	0.019	0,061	0	0	0	0	0	0	0
Frequency	217	0	0	0.006	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Frequency	220	0	0	0	0	0	0	0	0	0	0	0	0.038	0,061	0	0	0	0	0	0	0
Frequency	223	0	0	0	0	0	0	0	0	0	0	0	0.019	0,015	0	0	0	0	0	0	0
Frequency	229	0	0	0.006	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Frequency	235	0	0	0	0	0	0	0	0	0	0	0	0	0,015	0	0	0	0	0	0	0
Frequency	238	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Frequency	241	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Proportion of heterozygotes		0.468	0.545	0.775	0.567	0	0	0	0.04	0	0	0	0.808	0.878	0.5	0.37	0.667	0.143	1	0.5	0
Gene Diversity (Nei)		0.495	0.648	0.783	0.71	0	0	0	0.04	0	0	0	0.84	0.884	0.433	0.349	0.529	0.143	0.942	0.857	0

L58 Populations :		vesP6	vesP5	vesF6	vesF5	spiLP6	spiLP5	spiLF6	spiLF5	spiHP6	spiHP5	spiHF6	serF6	serF5	hybP6	hybP5	hvspF6	HvspF5	hvseF6	HsevF5	HsespF5
Number of individuals		47	77	89	30	34	27	48	51	16	16	19	26	67	8	27	9	7	8	4	2
Number of Alleles		4	3	9	5	1	1	2	1	1	1	1	21	31	2	3	3	3	6	4	2
Frequency	105	0	0	0.011	0.017	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Frequency	107	0.159	0.188	0.275	0.217	1	1	0.99	1	1	1	1	0.192	0,136	0.813	0.574	0.667	0.5	0.313	0.5	0
Frequency	109	0.682	0.766	0.275	0.317	0	0	0	0	0	0	0	0	0,008	0.188	0.407	0.222	0.286	0.313	0.25	0
Frequency	111	0.068	0	0.371	0.433	0	0	0.01	0	0	0	0	0.058	0,023	0	0	0.111	0.214	0.125	0	0
Frequency	113	0	0	0.017	0	0	0	0	0	0	0	0	0.058	0,091	0	0	0	0	0.063	0.125	0.5
Frequency	115	0.091	0.045	0.011	0	0	0	0	0	0	0	0	0.058	0,076	0	0.019	0	0	0	0	0
Frequency	117	0	0	0.022	0	0	0	0	0	0	0	0	0	0,053	0	0	0	0	0	0	0
Frequency	119	0	0	0.011	0.017	0	0	0	0	0	0	0	0.058	0,023	0	0	0	0	0.063	0	0
Frequency	121	0	0	0	0	0	0	0	0	0	0	0	0.038	0,076	0	0	0	0	0.125	0	0
Frequency	123	0	0	0	0	0	0	0	0	0	0	0	0.058	0,015	0	0	0	0	0	0	0
Frequency	125	0	0	0	0	0	0	0	0	0	0	0	0.019	0,015	0	0	0	0	0	0	0
Frequency	127	0	0	0	0	0	0	0	0	0	0	0	0.019	0,008	0	0	0	0	0	0	0
Frequency	129	0	0	0	0	0	0	0	0	0	0	0	0.038	0,038	0	0	0	0	0	0	0
Frequency	131	0	0	0	0	0	0	0	0	0	0	0	0.019	0,030	0	0	0	0	0	0	0.5
Frequency	133	0	0	0	0	0	0	0	0	0	0	0	0.077	0,038	0	0	0	0	0	0.125	0
Frequency	135	0	0	0.006	0	0	0	0	0	0	0	0	0.077	0,083	0	0	0	0	0	0	0
Frequency	137	0	0	0	0	0	0	0	0	0	0	0	0	0,076	0	0	0	0	0	0	0
Frequency	139	0	0	0	0	0	0	0	0	0	0	0	0.058	0,060	0	0	0	0	0	0	0
Frequency	141	0	0	0	0	0	0	0	0	0	0	0	0.019	0,030	0	0	0	0	0	0	0
Frequency	143	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Frequency	145	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Frequency	147	0	0	0	0	0	0	0	0	0	0	0	0	0,015	0	0	0	0	0	0	0
Frequency	149	0	0	0	0	0	0	0	0	0	0	0	0.038	0,008	0	0	0	0	0	0	0
Frequency	151	0	0	0	0	0	0	0	0	0	0	0	0	0,015	0	0	0	0	0	0	0
Frequency	153	0	0	0	0	0	0	0	0	0	0	0	0.038	0,015	0	0	0	0	0	0	0
Frequency	155	0	0	0	0	0	0	0	0	0	0	0	0.019	0	0	0	0	0	0	0	0
Frequency	159	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Frequency	163	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Frequency	165	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Frequency	167	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Frequency	171	0	0	0	0	0	0	0	0	0	0	0	0.019	0,008	0	0	0	0	0	0	0
Frequency	181	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Frequency	183	0	0	0	0	0	0	0	0	0	0	0	0.019	0,008	0	0	0	0	0	0	0
Frequency	203	0	0	0	0	0	0	0	0	0	0	0	0.019	0	0	0	0	0	0	0	0
Proportion of heterozygotes		0.591	0.455	0.629	0.567	0	0	0.021	0	0	0	0	0.769	0.935	0.375	0.556	0.556	0.429	0.625	0.75	1
Gene Diversity (Nei)		0.503	0.378	0.714	0.676	0	0	0.021	0	0	0	0	0.94	0.943	0.325	0.514	0.523	0.67	0.817	0.75	1

L78	Populations :	vesP6	vesP5	vesF6	vesF5	spiLP6	spiLP5	spiLF6	spiLF5	spiHP6	spiHP5	spiHF6	serF6	serF5	hybP6	hybP5	hvspF6	HvspF5	hvseF6	HsevF5	HsespF5
Number of individuals		47	77	89	30	34	27	48	51	16	16	19	26	67	8	27	9	7	8	4	2
Number of Alleles		7	7	13	11	5	3	1	4	2	1	1	12	19	4	8	8	5	6	5	4
Frequency	116	0	0	0	0	0	0	0	0	0	0	0	0	0,016	0	0	0	0	0	0	0
Frequency	119	0	0	0	0	0	0	0	0	0	0	0	0.02	0,016	0	0	0	0	0	0	0
Frequency	122	0.011	0	0.006	0	0.044	0	0	0	0.719	1	1	0	0,016	0	0.13	0.063	0	0.063	0	0
Frequency	125	0	0	0.006	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Frequency	131	0.065	0.052	0.034	0.017	0.029	0	0	0	0	0	0	0.06	0	0.214	0.093	0	0	0.125	0	0
Frequency	134	0.043	0	0.011	0.033	0	0	0	0	0	0	0	0	0	0.071	0.019	0	0.071	0	0	0
Frequency	137	0.087	0.045	0.011	0.083	0.897	0.944	1	0.959	0.281	0	0	0	0,008	0.429	0.296	0.5	0.571	0	0	0
Frequency	140	0	0	0.011	0.017	0	0	0	0	0	0	0	0.08	0,119	0	0	0	0	0	0	0
Frequency	143	0	0	0	0	0	0	0	0	0	0	0	0.08	0,024	0	0	0	0	0	0	0
Frequency	146	0	0.013	0.034	0	0	0	0	0.01	0	0	0	0.14	0,135	0	0.019	0.063	0	0	0	0.25
Frequency	149	0.011	0.058	0.125	0.183	0	0	0	0.02	0	0	0	0.14	0,270	0	0	0.063	0.143	0.375	0.125	0.25
Frequency	152	0.37	0.403	0.307	0.15	0.015	0.019	0	0	0	0	0	0.14	0,143	0	0.259	0.125	0.071	0.313	0.25	0.25
Frequency	155	0.413	0.383	0.256	0.283	0.015	0.037	0	0	0	0	0	0.2	0,111	0.286	0.13	0.063	0.143	0.063	0.375	0
Frequency	158	0	0	0.085	0.1	0	0	0	0.01	0	0	0	0.04	0,048	0	0	0.063	0	0.063	0.125	0.25
Frequency	161	0	0.045	0.091	0.1	0	0	0	0	0	0	0	0	0	0	0.056	0.063	0	0	0	0
Frequency	164	0	0	0.023	0.017	0	0	0	0	0	0	0	0.04	0,024	0	0	0	0	0	0	0
Frequency	167	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Frequency	170	0	0	0	0	0	0	0	0	0	0	0	0.02	0,016	0	0	0	0	0	0.125	0
Frequency	173	0	0	0	0.017	0	0	0	0	0	0	0	0	0,016	0	0	0	0	0	0	0
Frequency	176	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Frequency	179	0	0	0	0	0	0	0	0	0	0	0	0.04	0,016	0	0	0	0	0	0	0
Frequency	182	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Frequency	185	0	0	0	0	0	0	0	0	0	0	0	0	0,008	0	0	0	0	0	0	0
Proportion of heterozygotes		0.435	0.662	0.841	0.6	0.059	0.074	0	0.02	0.063	0	0	0.44	0.857	0.429	0.481	0.75	0.571	0.625	0.5	1
Gene Diversity (Nei)		0.686	0.685	0.811	0.849	0.195	0.108	0	0.08	0.417	0	0	0.897	0.865	0.736	0.814	0.758	0.67	0.783	0.857	1

L94	Populations :	vesP6	vesP5	vesF6	vesF5	spiLP6	spiLP5	spiLF6	spiLF5	spiHP6	spiHP5	spiHF6	serF6	serF5	hybP6	hybP5	hvspF6	HvspF5	hvseF6	HsevF5	HsespF5
Number of individuals		47	77	89	30	34	27	48	51	16	16	19	26	67	8	27	9	7	8	4	2
Number of Alleles		4	3	8	7	3	1	1	3	1	1	1	5	10	3	6	5	4	5	3	1
Frequency	130	0	0	0	0.017	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Frequency	136	0.011	0	0	0	0	0	0	0	0	0	0	0	0	0	0.037	0	0	0	0	0
Frequency	139	0	0	0	0	0.03	1	0	0	0	0	0	0	0,015	0	0.204	0	0	0	0	0
Frequency	145	0.181	0.117	0.006	0	0.924	0	1	0.961	1	1	1	0.16	0,104	0.375	0.222	0.444	0.286	0	0	1
Frequency	151	0	0	0.022	0.017	0	0	0	0	0	0	0	0	0,007	0	0	0.056	0	0	0	0
Frequency	154	0.447	0.5	0.607	0.617	0.045	0	0	0	0	0	0	0.18	0,201	0.438	0.315	0.389	0.429	0.375	0.25	0
Frequency	157	0	0	0	0	0	0	0	0	0	0	0	0	0,007	0	0.019	0	0	0	0	0
Frequency	160	0.362	0.383	0.185	0.183	0	0	0	0.02	0	0	0	0.54	0,530	0.188	0.204	0.056	0.143	0.438	0.625	0
Frequency	163	0	0	0.022	0.067	0	0	0	0	0	0	0	0.02	0,030	0	0	0	0	0.063	0	0
Frequency	166	0	0	0.056	0.067	0	0	0	0.02	0	0	0	0	0	0	0	0	0.143	0.063	0	0
Frequency	169	0	0	0.079	0	0	0	0	0	0	0	0	0	0,022	0	0	0.056	0	0	0	0
Frequency	172	0	0	0	0	0	0	0	0	0	0	0	0.1	0,067	0	0	0	0	0	0.125	0
Frequency	175	0	0	0.022	0.033	0	0	0	0	0	0	0	0	0,015	0	0	0	0	0	0	0
Frequency	178	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.063	0	0
Proportion of heterozygotes		0.638	0.558	0.573	0.533	0.03	0	0	0.059	0	0	0	0.52	0.661	0.625	0.63	0.778	0.571	0.625	0.5	0
Gene Diversity (Nei)		0.644	0.593	0.59	0.585	0.145	0	0	0.077	0	0	0	0.653	0.667	0.675	0.781	0.68	0.747	0.7	0.607	0

fsp1	Populations :	vesP6	vesP5	vesF6	vesF5	spiLP6	spiLP5	spiLF6	spiLF5	spiHP6	spiHP5	spiHF6	serF6	hybP6	hybP5	hvspF6	HvspF5	hvseF6	HsevF5	HsespF5
Nombre de gènes		92	154	176	60	68	54	96	100	32	32	38	52	14	54	18	14	16	8	4
Nombre d'Allèles		8	8	9	8	2	1	3	1	1	2	1	9	5	8	6	5	7	4	3
Fréquences	130	0	0	0	0	0	0	0	0	0	0	0	0.019	0	0	0	0	0	0	0
Fréquences	136	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fréquences	138	0.141	0	0	0	0	0	0.031	0	0	0	0	0.096	0	0.019	0	0	0	0	0
Fréquences	140	0.054	0.104	0.227	0.2	0.015	0	0.833	1	1	0.969	1	0.308	0.143	0.204	0.556	0.357	0.25	0.125	0.5
Fréquences	142	0.054	0.104	0.085	0.05	0.985	1	0.135	0	0	0.031	0	0.269	0.5	0.278	0	0.071	0.188	0	0.25
Fréquences	144	0.478	0.506	0.102	0.083	0	0	0	0	0	0	0	0.154	0.214	0.333	0.111	0.214	0.125	0.25	0
Fréquences	146	0.022	0.032	0.199	0.233	0	0	0	0	0	0	0	0	0.071	0	0	0	0.063	0	0
Fréquences	148	0.033	0.052	0.193	0.267	0	0	0	0	0	0	0	0.019	0	0.019	0.111	0.286	0	0.375	0.25
Fréquences	150	0.207	0.143	0.142	0.117	0	0	0	0	0	0	0	0.077	0.071	0.093	0.111	0.071	0.188	0.25	0
Fréquences	152	0	0.006	0	0	0	0	0	0	0	0	0	0	0	0.019	0	0	0.125	0	0
Fréquences	154	0	0	0.023	0.017	0	0	0	0	0	0	0	0	0	0	0	0	0.063	0	0
Fréquences	156	0	0	0	0	0	0	0	0	0	0	0	0.038	0	0	0	0	0	0	0
Fréquences	158	0	0	0.017	0.033	0	0	0	0	0	0	0	0.019	0	0	0.056	0	0	0	0
Fréquences	160	0	0	0.011	0	0	0	0	0	0	0	0	0	0	0	0.056	0	0	0	0
Fréquences	162	0.011	0.052	0	0	0	0	0	0	0	0	0	0	0	0.037	0	0	0	0	0
Proportion d'hétérozygotes		0.565	0.701	0.795	0.767	0.029	0	0.021	0	0	0.063	0	0.615	0.714	0.63	0.667	0.429	0.875	0.5	0.5
Gene Diversity de Nei		0.709	0.7	0.837	0.824	0.029	0	0.289	0	0	0.063	0	0.807	0.725	0.774	0.686	0.791	0.883	0.821	0.833

fsp2	Populations :	vesP6	vesP5	vesF6	vesF5	spiLP6	spiLP5	spiLF6	spiLF5	spiHP6	spiHP5	spiHF6	serF6	hybP6	hybP5	hvspF6	HvspF5	hvseF6	HsevF5	HsespF5
Nombre de gènes		62	154	176	60	60	54	96	102	32	32	38	44	12	54	18	12	12	4	2
Nombre d'Allèles		6	4	6	7	1	2	1	3	2	1	3	4	1	4	3	3	4	3	1
Fréquences	152	0	0	0.011	0	0	0	0	0	0	0	0	0.023	0	0	0	0	0.083	0	0
Fréquences	154	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0.083	0	0	0
Fréquences	156	0.339	0.539	0.324	0.15	1	0.963	1	0.971	0.906	1	0.368	0.75	1	0.796	0.667	0.667	0.583	0.25	1
Fréquences	160	0.113	0.039	0.142	0.15	0	0	0	0.01	0	0	0	0.023	0	0.037	0.056	0	0	0.5	0
Fréquences	164	0.339	0.351	0.472	0.533	0	0.037	0	0.02	0.094	0	0	0.205	0	0.13	0.278	0.25	0.25	0.25	0
Fréquences	166	0.113	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fréquences	168	0	0	0.006	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fréquences	176	0.081	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fréquences	182	0.016	0	0	0.017	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fréquences	184	0	0	0	0.017	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fréquences	192	0	0.071	0.045	0	0	0	0	0	0	0	0.605	0	0	0.037	0	0	0.083	0	0
Fréquences	194	0	0	0	0.083	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fréquences	198	0	0	0	0	0	0	0	0	0	0	0.026	0	0	0	0	0	0	0	0
Proportion d'hétérozygotes		0.258	0.208	0.557	0.567	0	0	0	0.039	0.063	0	0.263	0.455	0	0.185	0.444	0.333	0.667	1	0
Gene Diversity de Nei		0.75	0.584	0.654	0.672	0	0.073	0	0.058	0.175	0	0.511	0.404	0	0.353	0.503	0.53	0.636	0.833	0

APPENDIX V

During this thesis, several crosses were tried.

Individual crosses were performed at first.

Oogonia of *F. spiralis* and *F. vesiculosus* were isolated by collecting them from conceptacles after dissection with a razor blade. Clusters of antheridia were obtained in the same manner.

Crosses were performed in sterile plastic dishes containing 3ml sterile seawater at 5°C. The different sets of crosses are presented in Figure1. Two days later, saewater was replaced by culture medium (Provali's Enriched Seawater) containing 6 $mg.l^{-1}$ GeO_2 to prevent growth od diatioms. Replenishment of seawater occurred at two weeks intervals, but without GeO_2.

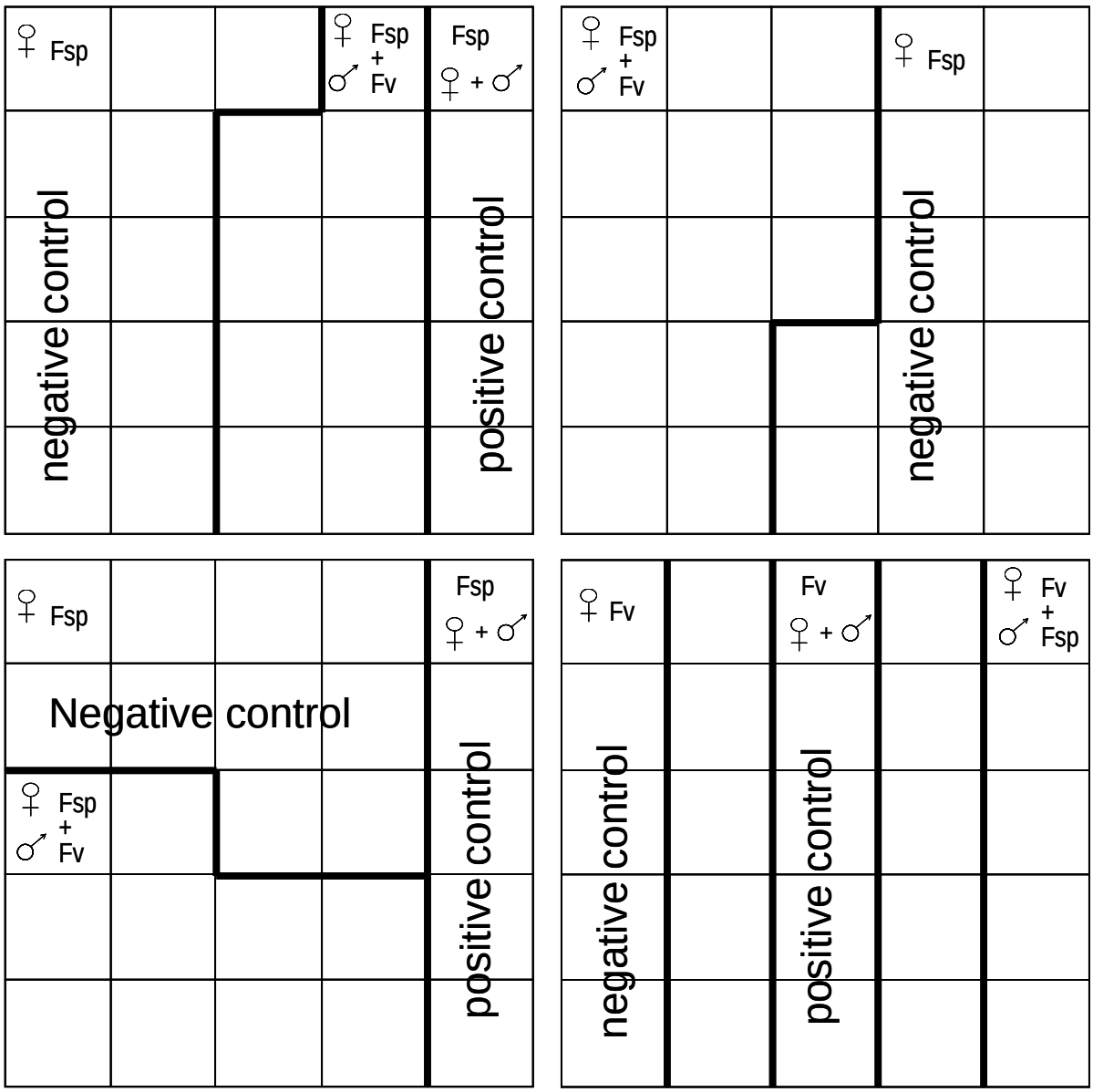

Figure 1: four plates of crosses experiment. Each square represents an individual cell. The type of cross is given in the first cell and the different types of crosses are shown by bold lines

The second protocol used to perform the crosses was to let the receptacles spawning. Receptacles were cut and let one hour at sunlight to let them dry. They were put in dishes containing artificial seawater (table 1) to release their gametangia. To collect oogonia from the hermaphroditic *F. spiralis*, receptacles were put in high K^+ Seawater (K^+ASW) to prevent self fertilization. K^+ASW and gametangia were then passed through a 50µm nylon mesh (retaining oogonia but not antheridia), which was rinsed with normal seawater and eggs were collected from the mesh. To collect antheridia, receptacles released in normal seawater and antheridia were collected through the 50 µm nylon mesh. Mixes of antheridia and oogonia were done in Petri dishes. The different crosses are given in tables 2a, 2b, 2c and 2d.

The growth of individuals was followed during several months (Figure 2) but after the first two months, they did not grow anymore.

Table 1: Artificial seawater receipt

		normal seawater		**high K+ seawater**	
Salts:	FW	Molarity (mM)	for 1 liter	Molarity (mM)	for 1 liter
NaCl	58,44	450	26,30	0	0,00
MgCl2	203,3	30	6,10	30	6,01
MgSO4	246,47	16	3,94	16	3,94
CaCl2	219,08	9	1,97	9	1,97
	147,02	9	1,32	9	1,32
	110,99	9	1,00	9	1,00
KCl	74,55	12	0,89	462	34,44
NaHCO3	84,01	0	0,00	0	0,00

Inter-specific crosses

Table 2a: Crosses between eggs from *F. vesiculosus* with sperm from *F. spiralis*. X indicate which crosses were tried, for example, eggs from Fv1 were mixed with the sperm of Fsp1 in one case and with a mixture of sperm of all *F. spiralis* individuals

		Females				
		Fv1	Fv2	Fv3	Fv4	mixture
Males	Fsp1	**X**				
	Fsp2		**X**			**X**
	Fsp3			**X**		
	Fsp4				**X**	**X**
	mixture	**X**		**X**		

Table 2b: Crosses between eggs from *F. spiralis* with sperm from *F. vesiculosus*.

		Females				
		Fsp1	Fsp2	Fsp3	Fsp4	mixture
Males	Fv1	**X**				**X**
	Fv2		**X**		**X**	
	Fv3			**X**		**X**
	mixture			**X**	**X**	

Intra-specific crosses

Table 2c: Crosses between eggs from *F. spiralis* with sperm from *F. vesiculosus*.

		Females			
		Fsp1	Fsp2	Fsp3	Fv4
Males	Fsp1	**X**			
	Fsp2		**X**	**X**	
	Fsp3		**X**	**X**	
	Fv4				**X**

Negative controls

Table 2d: culture of eggs from *F. spiralis* without sperm, to check the absence of self-fertilization.

	Females			
	Fsp1	Fsp2	Fsp3	Fsp4
0 Male	**X**	**X**	**X**	**X**

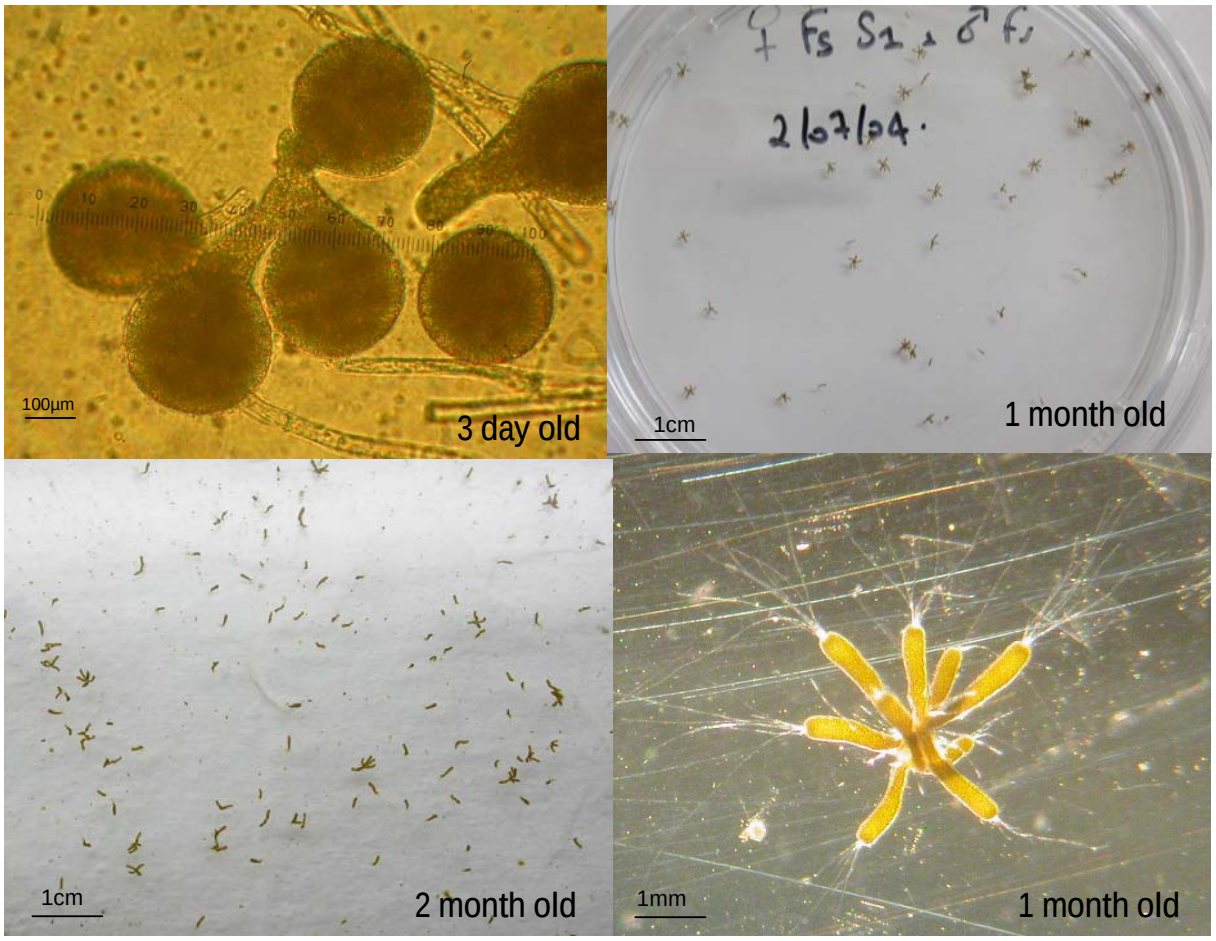

Figure 2: Picture of individuals after 3 days, one month and two months. 2a: apical growth of the rhizoids. 2b, 2c, 2d: Eggs coming from the same antheridia can stay attached and grow together after fertilisation.