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GENETIC ISOLATION BETWEEN THREE CLOSELY RELATED TAXA: *FUCUS VESICULOSUS, F. SPIRALIS*, AND *F. CERANOIDES* (PHAOPHYCEAE)[1]

Emmanuelle Billard, Claire Daguin

Equipe Evolution et Génétique des Populations Marines, UMR 7144 CNRS/UPMC, Station Biologique de Roscoff, B.P.74, Place Georges Teissier, 29682 Roscoff cedex, France, and CCMAR/CIMAR–Laboratório Associado, FCMA, Universidade do Algarve, 8005–139 Faro, Portugal

Gareth Pearson, Ester Serrão

CCMAR/CIMAR–Laboratório Associado, FCMA, Universidade do Algarve, 8005–139 Faro, Portugal

Carolyn Engel and Myriam Valero[2]

Equipe Evolution et Génétique des Populations Marines, UMR-CNRS/UPMC 7144, Station Biologique de Roscoff, B.P.74, Place Georges Teissier, 29682 Roscoff cedex, France

All traditional markers, both phenotypic and phylogenetic, have failed to discriminate between the taxa composing the *Fucus vesiculosus* L., *F. spiralis* L., and *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 versus 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 support 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*.

Key index words: **fucoid; genetic differentiation; genetic taxonomy; microsatellite; Phaeophyceae; species complex**

Abbreviations: **CA, correspondence analysis; UPGMA, unweighted pair group method using an arithmetic average**

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 (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 differentiated 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 vs. upper shore or rocky shores vs. 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 (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 internal transcribed spacer 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 internal transcribed spacer 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 used for taxonomic purposes, these markers have been shown to be useful for distinguish-

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[2]Author for correspondence: e-mail valero@sb-roscoff.fr.

TABLE 1. Sampled populations of the three *Fucus* taxa.

Species	Code	Location	Geoposition	Sample size
F. spiralis	FsSant1	Santec (NB)[a]	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)[b]	47°55′N, 3°70′E	24
	FcSLau2	Saint Laurent (SB)	47°55′N, 3°70′E	24

At each location, the distance between replicate samples was between 15 and 150 m. *Fucus vesiculosus* and *F. spiralis* samples from Santec and Brignogan were collected as attached to rocks in open coastal habitats, whereas all other samples were collected in muddy estuaries. NB, north Brittany; SB, south Brittany.

ing closely related species (Muir et al. 2000). Indeed different microsatellite markers were used recently to distinguish estuarine forms of *F. vesiculosus* and *F. spiralis* (Wallace et al. 2004).

MATERIALS AND METHODS

Sampling. Twenty populations were sampled in the Brittany region, France (Table 1) to investigate whether genetic distances were greater between taxa, between habitats (estuary vs. 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. *Fucus vesiculosus* was therefore sampled from both open coast and estuarine locations 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 kilometers 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 (two to three for each individual) were stored in silica gel for future molecular analyses.

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

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 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 and unweighted pair group method using an arithmetic average (UPGMA) using PHYLIP software (Felsenstein 1986). Robustness of the topology was tested using 1000 bootstrap resamplings. Because 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 (Sites and Marshall 2004). 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).

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

TABLE 2. Allele frequencies at five microsatellite loci.

Locus 20				Locus 58				Locus 94				Locus 38				Locus 78			
Allele size	*Fsp* $n = 131$	*Fve* $n = 226$	*Fce* $n = 84$	Allele size	*Fsp* $n = 136$	*Fve* $n = 220$	*Fce* $n = 79$	Allele size	*Fsp* $n = 136$	*Fve* $n = 219$	*Fce* $n = 75$	Allele size	*Fsp* $n = 136$	*Fve* $n = 211$	*Fce* $n = 89$	Allele size	*Fsp* $n = 136$	*Fve* $n = 195$	*Fce* $n = 83$
120	—	0.013	—	105	—	0.014	0.000	127	—	0.002	—	139	—	0.002	—	113	—	0.005	—
129	—	0.002	—	107	1.000	0.357	0.006	136	—	0.005	—	163	—	0.019	—	119	—	0.005	—
135	—	0.004	—	109	—	0.296	0.013	145	0.996	0.018	—	166	—	0.010	—	122	0.956	0.010	—
138	—	0.124	0.006	111	—	0.314	0.032	148	—	0.034	—	169	1.000	0.251	—	128	—	0.005	—
141	0.004	0.126	—	113	—	0.014	0.006	154	0.004	0.434	1.000	172	—	0.043	—	131	—	0.013	1.000
144	—	0.175	—	115	—	0.002	0.817	157	—	0.007	—	175	—	0.005	—	132	0.011	—	—
147	0.920	0.086	0.738	117	—	0.002	0.127	160	—	0.279	—	181	—	0.010	—	134	—	0.005	—
150	—	0.049	0.060	119	—	0.002	0.000	163	—	0.027	—	190	—	0.495	0.994	137	0.022	0.021	—
153	0.076	0.208	0.179					166	—	0.135	—	193	—	0.031	—	140	—	0.005	—
156	—	0.144	0.018					169	—	0.048	—	196	—	0.026	0.006	143	—	0.056	—
159	—	0.031	—					175	—	0.011	—	199	—	0.088	—	146	—	0.003	—
162	—	0.022	—									205	—	0.002	—	149	—	0.123	—
165	—	0.002	—									208	—	0.012	—	152	—	0.226	—
168	—	0.002	—									211	—	0.005	—	155	0.011	0.190	—
171	—	0.007	—									226	—	0.002	—	158	—	0.051	—
174	—	0.002	—													161	—	0.241	—
180	—	0.002	—													164	—	0.028	—
																167	—	0.010	—
																176	—	0.003	—

Frequencies were computed for the total sample within each of the three species: *Fucus spiralis* (*Fsp*), *F. vesiculosus* (*Fve*), and *F. ceranoides* (*Fce*). *n*, sample size.

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, because of 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 and 154 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 internal transcribed spacer sequences failed to 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, whereas 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). 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 this taxon at the diagnostic locus 78, whereas 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*.

The UPGMA and neighbor-joining phylogenetic reconstruction methods (Fig. 2, A and B, 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 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

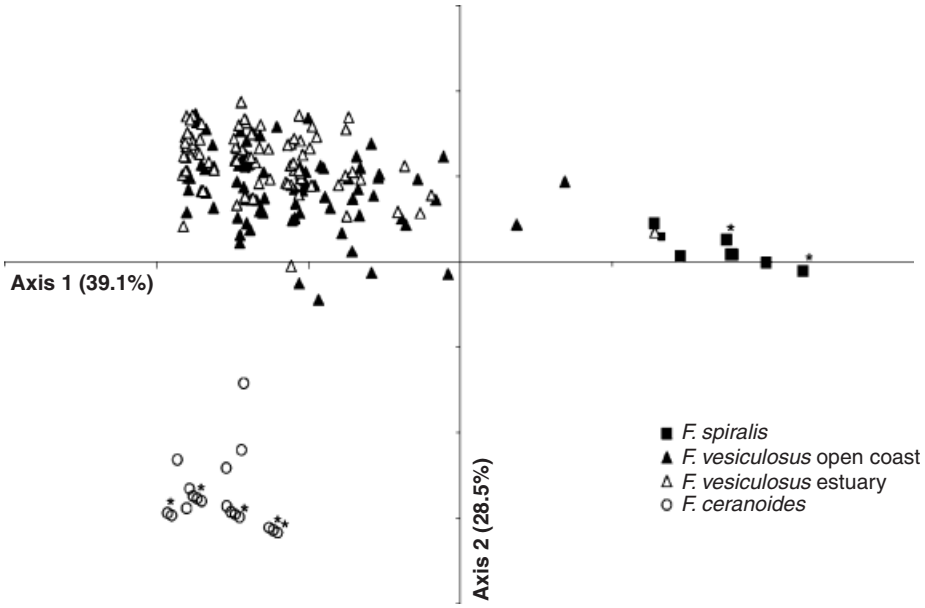

FIG. 1. CA based on allele frequencies of *Fucus spiralis*, *F. vesiculosus*, and *F. ceranoides* populations in Brittany calculated at five microsatellite loci. Plot of individuals. Inertia of each axis is given in parentheses. Asterisk indicates more than one individual superimposed.

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).

In the neighbor-joining tree, two well-defined clades (bootstrap values >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 scatter-

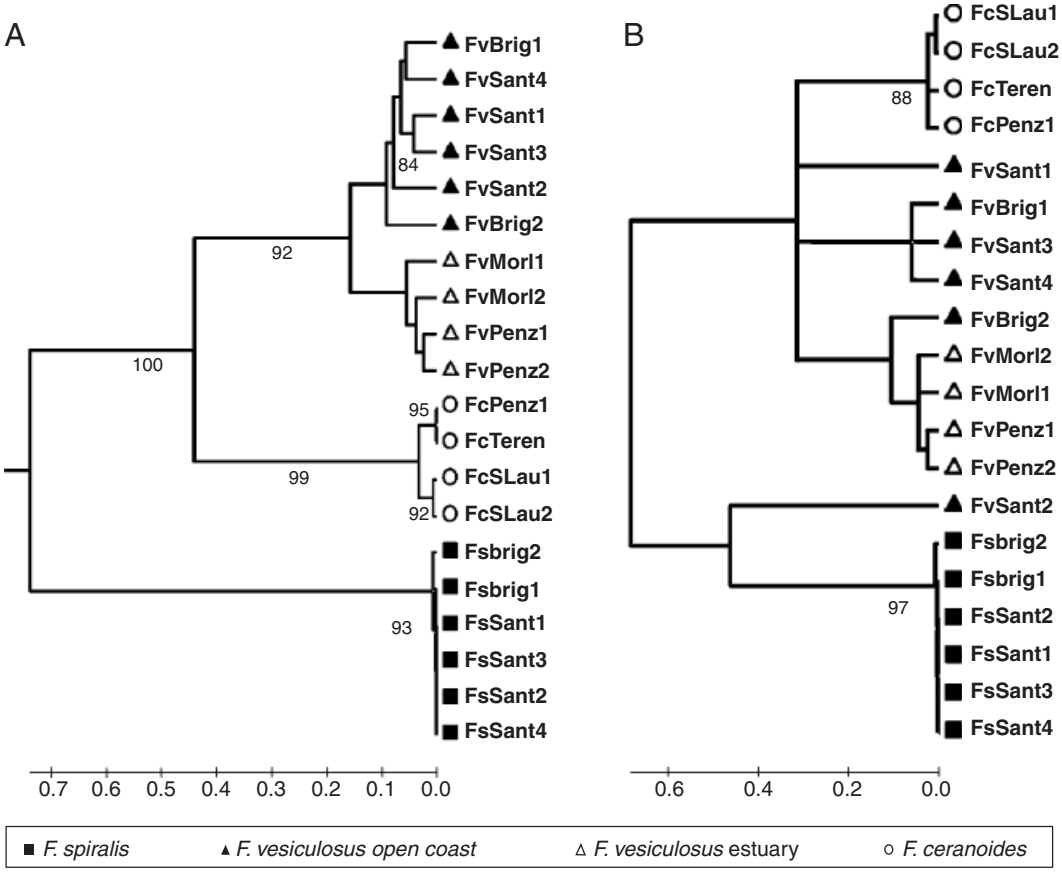

FIG. 2. (A) UPGMA and (B) neighbor-joining distance tree of populations of *Fucus spiralis*, *F. vesiculosus*, and *F. ceranoides* based on Nei's distances (see scale bar). Only bootstrap values superior or equal to 80% from 1000 replicates are shown.

ing of *F. vesiculosus* populations through the tree revealed that this taxon is not as genetically cohesive as the other two taxa. Like the CA, 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* was recently demonstrated in the northwest Atlantic (Maine, USA, Wallace et al. 2004) and in two distant regions of the northeast Atlantic coast (Cape Gris-Nez in northern France and Viana do Castelo in northern Portugal, Engel et al. 2005). 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 approximately 200 to 300 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 versus 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 (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. 2005), we suggest that reproductive isolation may not be complete between *F. 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 algal model 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) versus mosaic hybrid zones between *F. vesiculosus* and *F. spiralis* (Wallace et al. 2004, Engel et al. 2005, 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.

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