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Hybridization between two cryptic filamentous brown seaweeds along the shore: Analysing pre- and post-zygotic barriers in populations of individuals with varying ploidy levels.

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Abstract:

We aimed to study the importance of hybridization between two cryptic species of the genus *Ectocarpus,* a group of filamentous algae with haploid-diploid life cycles that include the principal genetic model organism for the brown algae. In haploid-diploid species, the genetic structure of the two phases of the life cycle can be analysed separately in natural populations. Such life cycles provide a unique opportunity to estimate the frequency of hybrid genotypes in diploid sporophytes and meiotic recombinant genotypes in haploid gametophytes allowing the effects of reproductive barriers preventing fertilization or preventing meiosis to be untangle. The level of hybridization between *E. siliculosus* and *E. crouaniorum* was quantified along the European coast. Clonal cultures (568 diploid, 336 haploid) isolated from field samples were genotyped using cytoplasmic and nuclear markers to estimate the frequency of hybrid genotypes in diploids and recombinant haploids. We identified admixed individuals using microsatellite loci, classical assignment methods and a newly developed Bayesian method (*XPloidAssignment*), which allows the analysis of populations that exhibit variations in ploidy level. Over all populations, the level of hybridization was estimated at 8.7%. Hybrids were exclusively observed in sympatric populations. More than 98% of hybrids were diploids (40% of which showed signs of aneuploidy) with a high frequency of rare alleles. The near absence of haploid recombinant hybrids demonstrates that the reproductive barriers are mostly post-

zygotic and suggests that abnormal chromosome segregation during meiosis following hybridisation of species with different genome sizes could be a major cause of interspecific incompatibility in this system.

Introduction

Hybridization has been defined by Barton & Hewitt (1985) as reproduction between individuals from genetically different populations. Hybridization implies sexual reproduction, which in turn entails cyclic alternation between syngamy and meiosis. However, the relative timing of syngamy and meiosis varies widely across the eukaryotic tree of life (Valero *et al.* 1992; Mable & Otto 1998; Coelho *et al.* 2007). For example, syngamy and meiosis are clearly separated in time in haploid–diploid species. In such life cycles, there is an alternation between haploid individuals (gametophytes) produced by meiosis and diploid individuals (sporophytes) resulting from syngamy. Haploid-diploid life cycles are widespread in red, brown and green algae and in mosses and ferns. Unlike diploid-dominant plants and animals, the haploid stage in a haploid-diploid life cycle is an independent, functional organism often with extensive somatic development (reviewed in Valero et al. 1992; Mable & Otto 1998). Consequently, in haploid-diploid species the two hallmarks of the sexual life cycle can be studied separately by analysing the population genetic structure of the haploid gametophytes and the diploid sporophytes. In particular, the importance of post-zygotic barriers in the wild may be easily estimated by comparing diploid and haploid subpopulations (i.e. by estimating the frequency of hybrid genotypes in diploid sporophytes and meiotic recombinant genotypes in haploid gametophytes) (Fig. 1). Direct access to the haploid part of the life cycle allows the effects of reproductive barriers that prevent fertilization (i.e. prevent the formation of diploid hybrid genotypes) to be distinguished from those that prevent meiosis (i.e. prevent the formation of recombinant haploid genotypes) (Fig. 1). In diploid species, these processes cannot be distinguished directly using population genetics in the field (Fig. 1). Investigating a large diversity of biological models

is thus crucial to unravel the general importance of hybridization in evolution. However, most studies of hybridization in the wild have been carried out on flowering plants or animals. It is only recently that data are accumulating for species belonging to other phyla such as fungi (see for reviews Kohn 2005; Giraud *et al.* 2008), red algae (Zuccarello *et al.* 2005; Destombe *et al.,* 2010; Maggs *et al.* 2011; Niwa & Kobiyama 2014; Savoie & Saunders 2015; Guillemin *et al.* 2016) and haploid-diploid brown algae (Peters *et al.* 2010a; Geoffroy *et al.* 2015 and references therein), which differ substantially in their life cycle and life history traits from flowering plants and animals.

Since the publication of the *Ectocarpus* genome sequence by Cock *et al.* in 2010, species from this genus have been used as model systems in various research fields and, in particular, because of their haploid-diploid life cycle (Müller 1967), in developmental genetics (Cock *et al.* 2014). *Ectocarpus* spp. are ephemeral algae. These brown, filamentous algae have been described as short-lived annuals, found on abiotic substrata or epiphytic on macrophytes, along the shore gradient from subtidal up to high intertidal pools (Russell 1967a, b, 1983). They are easy to cultivate in the laboratory but, until recently, genetic population studies were challenging since these small organisms are difficult to sample and even observe in the field. In order to have access to microscopic individuals and to obtain a sufficient amount of DNA, uniclonal culture has to be performed in the laboratory prior to any genetic analyses (Couceiro *et al.* 2015). Since the development of genetic and genomic tools for this algal model, several studies have been undertaken in the field to better understand their ecology and diversity (Peters *et al.* 2010a, b; Couceiro *et al.* 2015; Peters *et al.* 2015; Montecinos *et al.* in press). From these studies, it appears that the genus *Ectocarpus* forms a complex of cryptic sibling species adapted to different hosts and/or habitats along the shore gradient. Moreover, incongruencies between a nuclear (internal transcribed spacer 1 of the ribosomal DNA; ITS1) and a cytoplasmic marker (cytochrome oxidase subunit 1; COI-5P) were recently reported by Montecinos *et al.* (in

press) among some species pairs suggesting the occurrence of hybridization and introgression in the field. Also, the occurrence of putative hybrids between *E. siliculosus* and *E. crouaniorum* in the Atlantic coasts was reported by Peters *et al.* (2010a), who used a barcode approach based on ITS1 length difference. Individual species can be rapidly identified based on ITS length and individuals bearing both species-specific bands were regarded as putative hybrids (Peters et al. 2010a). However, although *E. siliculosus* and *E. crouaniorum* were the most frequently sampled species along the North Atlantic coast by Montecinos et al. (in press), no incongruences between the nuclear and cytoplasmic markers were reported between these two species by these authors. These results question the importance of hybridization in nature between *E. siliculosus* and *E. crouaniorum* and might indicate the occurrence of post-zygotic barriers preventing gene flow and introgression between these species.

To address the importance and nature of reproductive barriers in the haploid-diploid *Ectocarpus* genus, we completed a collection of more than 900 unialgal cultures derived from samples of *E. siliculosus* and *E. crouaniorum* collected in ten sites along the European coast. We first combined information from cytoplasmic and nuclear markers to estimate the frequency of each parental species and putative hybrids at the different sites along the European coast. We then analysed microsatellite genetic data to conduct admixture analyses with the classical STRUCTURE and GENECLASS methods. One drawback of these clustering models is that they must be applied separately to haploid and diploid sub-populations. We thus formalized a new Bayesian method, which allows the quantification of admixture between two cryptic species using all individuals whatever their ploidy (ie haploid, diploid or aneuploid). The objectives of this study were (1) to test whether hybrids were restricted to sympatric zones where the two species are in contact along the shore, and (2) to determine the relative abundance of haploid and diploid hybrids and describe their genetic composition. In addition to contributing important information concerning the biology of *Ectocarpus*, this study aimed to broaden our understanding of speciation and hybridization processes in benthic marine environments focusing for the first time on organisms with complex haploid-diploid life cycles.

Material and Methods

Field collections, isolation of Ectocarpus *strains and DNA extraction*

A total of 904 *Ectocarpus* samples from nine sites located along the North East Atlantic (NEA) coast and one site located on the Mediterranean Sea were used in this study (Fig. S1, supporting information). Four hundred and ninety-four samples were collected from seven sites along the NEA in the framework of this study, while the remaining 410 samples (Roscoff and Naples sites) were previously obtained by Couceiro et al. (2015). As *Ectocarpus* species cannot be distinguished in the field (see below), sampling was blind and it was not possible to select, a priori, sites where the two species co-occurred in sympatry. Nonetheless, since *E. siliculosus* and *E. crouaniorum* have been shown to inhabit different levels of the intertidal (Peters *et al.* 2010a; Couceiro *et al.* 2015; Montecinos *et al.* in press), whenever possible, samples were collected from high to lower tide levels on the shore. Unialgal cultures were established from all 494 newly collected samples and maintained as clonal cultures using the protocol described in Couceiro *et al.* (2015). After two months of laboratory culture, enough biomass was available for DNA extraction and total DNA was extracted from lyophilized samples using the NucleoSpinR 96 Plant Kit (Macherey-Nagel, Duren, Germany).

Molecular determination of ploidy and sex

In *Ectocarpus*, sex is expressed during the haploid phase and is determined by two different sex-determining regions (Ahmed *et al.* 2014), corresponding to the two sexes. Both the male and female sex-determining regions are present in the diploid sporophytes (heterozygous for the sex locus) whereas the haploid gametophytes carry either the male or female allele (hemizygous for the sex locus). In order to determine the sex and the ploidy of our samples, we amplified the sex-specific regions following Couceiro *et al.* (2015). Positive/negative amplifications for male and female specific PCR primers were checked on 2% agarose gels stained with ethidium bromide.

Preliminary sorting of samples into parental species or putative hybrid categories using the rps14-atp8 spacer, the ITS1 and diagnostic microsatellite loci and alleles

Neither the two cryptic species *E. siliculosus* (hereafter Esil) and *E. crouaniorum* (hereafter Ecro) nor their putative hybrids can be distinguished using morphological characters (Peters *et al.* 2010a, b; Montecinos *et al.* in press). We therefore first amplified the species-specific mitochondrial *rps*14-*atp*8 spacer region developed by Couceiro *et al.* (2015) to classify each sample cytoplasm by its species of origin. Peters et al. (2004b) have demonstrated that mitochondria are maternally inherited in *Ectocarpus,* thus the potential direction of interspecific crosses was inferred using this marker. We then amplified the ITS1 and tested for linkage disequilibrium between this nuclear marker and the cytoplasmic marker *rps*14-*atp*8 to distinguish parental species from putative hybrids. The ITS1 fragments allow the two parental species to be distinguished based on the length of the amplified region (850 bp in Esil or 1100 bp in Ecro). Individuals were considered as putative hybrids if they bore the two ITS1 fragments (850bp and 1100bp) or showed incongruence between the mitochondrial and nuclear markers. Finally, nine nuclear microsatellite loci (Couceiro *et al.* 2015) were used to complete the preliminary identification of putative hybrids in our data set. The degree to which these loci are diagnostic for the parental species was evaluated using the individuals previously classified as Esil or Ecro based on the cytoplasmic information. Six species-diagnostic loci, defined as loci showing no cross-amplification between both parental species, were retained. All individuals showing amplification products for at least one diagnostic locus inconsistent with the expected species based on the cytoplasmic markers were added to the list of putative hybrids.

The species-specific mitochondrial *rps*14-*atp*8 spacer region was amplified as described in Couceiro *et al.* (2015). A fragment containing the ITS1 region together with 224 bp of the flanking 18S and 5.8S genes was amplified using the primers and PCR conditions described by Peters *et al.* (2010a). The presence/absence of *rps*14-*atp*8 spacer bands and the number and

length of the ITS1 fragments were determined on 2% agarose gels. The nine nuclear microsatellite loci were amplified using the primers and PCR conditions described by Couceiro *et al.* (2015). Microsatellite alleles were analysed in an ABI 3130 automated sequencer and scored manually using GeneMapper ® version 4 software (Life Technologies Corporation). Samples showing missing data were re-amplified up to three times. For each locus, the frequency of missing data was calculated independently in the individuals previously classified as Esil or Ecro based on the cytoplasmic information in order to defined species-diagnostic loci.

Because diagnostic loci do not amplify in both species, they generate missing data for one of the parental species and this non-amplification corresponds to a phylogenetic signal but should be distinguished from a null allele. The method used to distinguish missing data caused by species-diagnostic loci from missing data caused by null alleles is detailed in Supplementary Material (Supplementary File 1, Supporting information). Missing data caused by species-diagnostic loci were replaced by an artificial allele of an arbitrary size of 800bp.

Finally, for the three remaining microsatellite loci showing cross-amplification between both parental species, species-diagnostic alleles were defined as alleles showing different size ranges between Esil and Ecro. Allele frequency and allele size were computed with GENALEX (Peakall & Smouse 2012) independently in the individuals previously classified as Esil or Ecro based on the cytoplasmic information. We recorded some genotypes with 3 alleles per locus at one or more cross amplifying loci (23 diploid sporophytes, see results below). In all these cases, their genotypes at one given locus involved two alleles diagnostic of one species and one allele diagnostic of the second species. Because aneuploidy cannot be taken into account for most of the classical analyses, these individuals were re-categorised as diploid heterozygotes and, one allele diagnostic of each species was selected in order to retain information indicating admixture between species. The most frequent allele in each species gene pool was conserved in the re-categorised diploids. We henceforth refer to these adjusted genotypes as "recoded".

Statistical analyses of admixture levels

To gain insight into hybridization between Esil and Ecro, the nuclear microsatellite dataset was analyzed following four complementary methods. First, a Principal Component Analyses (PCA) of the multilocus genotypes was carried out using the R-package ADEGENET (Jombart 2008). Second, the Bayesian clustering method STRUCTURE v2.3.4 (Pritchard *et al.* 2000) was used to identify the number of genetically distinct clusters that maximize the likelihood of the data, and to assign the individuals to the clusters Esil or Ecro using only genetic information. It is important to note that, unlike multivariate analyses (such as PCA), Bayesian clustering methods rely on explicit models and assumptions such as random mating and absence of linkage disequilibrium, which are often difficult to verify and can restrict their applicability. In STRUCTURE, the number K of populations was estimated using a burn-in period of 10,000 and 100,000 MCMC replicates, applying the admixture model and correlated allele frequencies. Each run was replicated 10 times and a range of clusters (K) from 1 to 10 was tested. To verify that the K value that best fitted our data was K = 2 (two species), the ΔK statistic developed by Evanno *et al.* (2005) was calculated. Combined results from the independent runs were obtained using the greedy algorithm with 100,000 random input orders in CLUMPP (Jakobsson & Rosenberg 2007) before exporting the results to DISTRUCT (Rosenberg 2004) for viewing. The estimated membership coefficients Q for each individual in each cluster were calculated using the results obtained by CLUMPP and those individuals for which the secondary cluster represented more than 10% of the genome were regarded as putative hybrids (Vähä & Primmer 2006). Since PCA multivariate analyses and STRUCTURE allow neither different ploidy levels within the same dataset nor aneuploidy, both analyses were run on the haploid and diploid sub-populations separately using the recoded aneuploid genotypes.

The third procedure employed to analyse our microsatellite dataset involved a new Bayesian method specifically developed for this study, implemented in the software XPLOIDASSIGNMENT (Supplementary File 2, Supporting information). This method assigns genotyped individuals

with different ploidy levels, both between individuals and between markers in the same individual to quantitative levels of admixture between two cryptic species (Esil and Ecro in our study). The method is described in Supplementary File 3 (Supporting information). Briefly, this method uses genotyped individuals a priori considered as pure bred, to compute, for each marker, two differentiated gene pools. The likelihood that an allele belongs to one of the differentiated gene pools, for example to Esil or Ecro, is its frequency within this differentiated gene pool. Here, we used XPLOIDASSIGNMENT to compute the posterior probabilities that each putative hybrid belonged corresponding to five different scenarios of hybridisation between Esil and Ecro: (1) hybrids with an equal proportion of Esil and Ecro genomes, parsimoniously interpreted as potential F1; (2) hybrids with ≥ 0.75 Esil genome, interpreted as admixed individuals derived from backcrosses or repeated interbreeding with Esil; (3) hybrids with ≥ 0.75 Ecro genome, interpreted as admixed individuals derived from backcrosses or repeated interbreeding with Ecro; (4) individuals with ≥ 0.90 Esil genome, interpreted as part of the parental species Esil and (5) individuals with ≥ 0.90 Ecro genome, interpreted as part of the parental species Ecro. In order to define the genetic pools of the two parental species, Esil and Ecro, we constructed a data file combining both haploid and diploid samples but excluding the putative hybrids (as defined using the combination of *rps*14-*atp*8 spacer, ITS1 and microsatellite diagnostic loci). As XPLOIDASSIGNMENT allows the use of samples with varying ploidy levels, aneuploid genotypes were included with no recoding.

In the fourth method, we used the function "HYBRIDIZE" included in the R-package ADEGENET (Jombart 2008) to simulate 500 artificial genotypes for the three hybrid scenarios previously mentioned (i.e. F1, backcross with Esil and backcross with Ecro) using the parental species dataset as input (i.e. after excluding putative hybrids as defined using the *rps*14-*atp*8, ITS1 markers and diagnostic loci). This procedure was carried out to compare the simulated hybrid genotypes with the genotypes of the putative hybrids collected in the field. We then used

GENECLASS v2.0 (Piry & Cornuet 1999) to assign all putative hybrids to one of the five groups described above (i.e. the two parental species and the three simulated hybrid classes). The Rannala & Mountain (1997) assignment algorithm was used as criterion for computation following the methods outlined in Paetkau et al. (2004) and the assignment probabilities of all putative hybrids to each group were tested using the ''assign or exclude individuals'' option (p=0.05). Since both ADEGENET and GENECLASS analyses are limited to diploid samples, only the diploid dataset together with the recoded aneuploid genotypes could be used for this fourth approach.

Results from STRUCTURE, XPLOIDASSIGNMENT and GENECLASS were compared and combined to classify each putative hybrid into the five different genetic classes described above. When using GENECLASS or XPLOIDASSIGNMENT methods, individuals were classified into a single hybrid class category (i.e. the one showing the maximum posterior probability) if the probability of assignment to this category was at least twice as high as the probability of assignment to any other category; otherwise, individuals were assigned to the two or three equally probable categories.

Population structure and mating system

As asexual reproduction (by vegetative fragmentation or via mitospores, Knight 1930) has been observed in *Ectocarpus*, the degree of clonality was assessed at each site in both haploid and diploid subpopulations in Esil and Ecro. The number of repeated multilocus genotypes was calculated and the genotypic diversity R was computed by dividing the number of distinct genotypes (G) by the number of individuals, corrected for sample size (i.e. R = G-1/N-1, Dorken & Eckert 2001) using GENECLONE 2.0 v6.41 (Arnaud-Haond & Belkhir 2007). In addition, to explore the departure from random mating in each diploid sub-population, F_{IS} values were calculated using the software GENETIX v4.05 (Belkhir *et al.* 2004). Significance of departure from random mating was tested by running 1,000 permutations of alleles among individuals within samples. Finally, in order to study the importance of genetic differentiation among sites

within each parental species (i.e. excluding potential hybrids), the global F_{ST} statistic (as defined by Weir & Cockerham 1984) was calculated independently for the haploid and diploid subpopulations of each species using GENETIX v4.05 (Belkhir *et al.* 2004). The level of significance was estimated using 1,000 permutations.

Results

Species identification and detection of putative hybrids

Out of a total of 904 individuals, the mitochondrial species-specific marker *rps*14-*atp*8 identified 505 individuals as Esil and 340 as Ecro. The taxonomic identity of these 845 samples was confirmed by the amplification of their ITS1 nuclear marker (Table 1). The sex-specific markers indicated that, for Esil, diploid sporophytes (347) were more frequently sampled than haploid gametophytes (158) while the frequencies of the two kinds of individual were similar in Ecro (162 diploid sporophytes and 178 haploid gametophytes, Table 1). The remaining 59 samples exhibited incongruences between the nuclear and the cytoplasmic markers as all amplified both Esil (850bp) and Ecro (1100bp) ITS1 (Table 1). These 59 diploid sporophytes were thus classified as putative hybrids while none of the 336 haploid individuals fell into this category (Table1). Amplification success and allele size range for the nine microsatellite loci varied according to the cytoplasmic species identification. Four microsatellite loci were found to be diagnostic for Esil. For these four loci, the frequency of amplification was 0.94-0.99 when considering only individuals bearing an Esil cytoplasm, while it was only 0.12-0.13 for individuals bearing an Ecro cytoplasm (Table 2, Fig. S2, Supporting information). Similarly, two loci were found to be diagnostic for Ecro. These loci showed a frequency of amplification of 0.88-0.97 in individuals bearing an Ecro cytoplasm but less than 0.12 in individuals with an Esil cytoplasm. The remaining three loci amplified in both species but presented different allele size ranges (M-122-2, M-208, M-162-1; Table 2, Fig. S2, Supporting information). Species identification combining the data from the three types of marker (i.e. mitochondrial rps14-atp8,

nuclear ITS1, and nuclear microsatellite loci) increased the number of putative hybrid individuals to 81 (the genotypes of each putative hybrid for all the markers used is given in Table S1a, Supporting information). All 81 putative hybrids showed amplification products for at least one diagnostic locus that was not congruent with the cytoplasmic species identification (see Table S1a, Supporting information). Most putative hybrids were diploids (79 out of a total of 568 diploid sporophytes); only two putative recombinant hybrids were identified among the 336 haploid gametophytes.

Multivariate clustering of individuals

The results of the PCA analyses illustrate the genetic structure between the parental species and the putative hybrids for both diploid sporophytes (Fig. 2a) and haploid gametophytes (Fig. 2b). The first two axes of the PCA explained 7.47% and 12.10% of the genetic variability of diploid sporophyte and haploid gametophyte individuals, respectively. Whatever the ploidy phase under study (*i.e.* diploid sporophytes or haploid gametophytes), the PCA revealed a clear genetic differentiation between Esil and Ecro. Among the 568 studied diploid sporophytes, 69 of the 79 putative diploid hybrids were located between the Esil and Ecro groups (Table S1b, Supporting information). The 69 intermediate individuals included not only all the 59 putative diploid hybrids that were characterized by a double ITS1 band pattern but also 10 additional individuals identified as admixed only on the basis of the microsatellite locus information (Table 1, Table S1b, Supporting information). Among the 336 haploid gametophytes, only one of the two putative hybrids could be distinguished from the two parental clusters (Fig. 2b). Finally, the PCA provided support for the classification of 97% of the putative hybrids as hybrids.

Admixture analysis and assignation of putative hybrids to genetic categories

The clustering analysis performed with STRUCTURE confirmed that two was the optimal number of clusters for both the haploid and diploid datasets (ΔK method of Evanno *et al.* 2005; Fig. S3, Supporting information). The level of admixture for each individual within each population is given in Fig. 3a for diploid sporophytes and Fig. 3b for haploid gametophytes. The results of this Bayesian approach were very similar to those obtained with the multivariate PCA analysis: the same 67 putative diploid hybrids (of 79 total) were assigned as admixed with both this method (Fig. 3a) and the PCA analysis (Table S1b, Supporting information). However, in contrast to the previous methods, this Bayesian approach was not able to identify any putative hybrids among the haploid gametophyte individuals (Fig. 3b). Finally, STRUCTURE results clearly showed that all putative hybrids were only observed in sympatric populations (Fig. 3a).

In order to better classify the putative hybrids, the observed diploid genotypes were compared with those simulated by ADEGENET HYBRIDIZE (Jombart 2008) using a graphical representation (Fig. 4). Assignment probabilities of the putative hybrids to each genetic class computed by GENECLASS are given in Table S1b (Supporting information). The first two axes of the PCA explained 3.85% of the total genetic variability. The simulated F1 were easily recognisable by their intermediate position between the two parental species while simulated backcrosses overlapped partly with the simulated F1 hybrids and their respective parental species (Fig. 4). On the Y axis, three points are well separated from other observed and simulated data (Fig.4). These corresponded to five individuals with three different multi-locus genotypes from the Ribadeo population (GAL 176, 177, 217, 237 and 239, Table S1a, Supporting information) that share the same rare diagnostic allele (allele 298 at locus M-162-1). Most of the 79 putative hybrid diploid sporophytes (54) were classified as simulated F1 by GENECLASS (Table S1b, Supporting information). Five hybrid individuals were assigned to the backcross with Esil class and 14 as to the backcross with Ecro class (Table S1b, Supporting information).

Our new method XPLOIDASSIGNMENT allowed a simultaneous analysis of all the 81 putative hybrids regardless of their ploidy and without the need to recode aneuploid genotypes. It identified the highest number of hybrids (79 / 81) compared to the other analyses described above. Only half of the 79 putative hybrids (43 diploids and 2 haploids) could be assigned to a single hybrid class with confidence (Table S1b, Supporting information). Twenty-six diploid sporophytes were assigned to the F1 hybrid class, 11 to the backcross with Esil class and six to the backcross with Ecro class (Table S1b, Supporting information). The two recombinant haploid hybrids were classified as backcrosses (Table S1b, Supporting information).

Comparison among methods and frequency of hybridization in populations

The four methods were mostly congruent with regards to distinguishing admixed individuals from parental species. Since two putative hybrids were consistently classified as parental species by all methods, we finally considered that 79 hybrids (77 diploids and 2 haploids) were present in our data set (Table S1b, Supporting information). However, classification of individuals into the different hybrid classes was challenging. The information provided by the different methods was thus combined to classify hybrids into one or more than one hybrid categories when methods were not congruent (Table S1b, Supporting information). The objective was to roughly distinguish recently formed hybrids from hybrids that had undergone subsequent generations of backcrosses. When we considered the 20 hybrids that were classified into a single category, most of these hybrids (19) were classified as recently formed (F1) while only one was classified as backcrossed (Tables S1b, Supporting information). Fifty seven individuals were assigned to two hybrid categories, the majority of them (45/57) were classified as potential F1 or backcrosses with either Esil (36) or Ecro (9) (Tables S1b, Supporting information).

Thirty-five per cent of the alleles genotyped in the 79 putative hybrids were rare alleles (i.e. observed at a frequency of less than 0.04 in the parental species, Table S1a, Supporting information). A total of 96 rare alleles were observed, 79 were shared between the parental species and the hybrids while 17 were only present in hybrids. Moreover, of the 79 shared rare alleles, two alleles (allele 307 of locus M-420 and allele 207 of locus M-239-3, Table S1a, Supporting information) increased from a frequency of less than 0.04 in parental species to frequency higher than 0.24 in hybrids.

Finally, more than 70% of the hybrids (58/79) possessed Ecro cytoplasmic markers (Table S1b, Supporting information). The percentage of admixed individuals over the whole data set including haploid and diploid subpopulations was estimated at 8.7% (79/904). The percentage of hybrids was much higher in diploid (77/568 =13.6%) than in haploid (2/336 = 0.6%) subpopulations. The occurrence of hybrids varied among populations and regions (from 0 to 0.33) and hybrids were only observed in sympatric populations (Table 3). The percentage of admixed haploid and diploid individuals, when calculated as a proportion of all the individuals in sympatric populations, was 11.5% (79/689).

Population structure and mating system

The efficiency of the nine microsatellite loci to discriminate individuals varied between species and among populations. Less than 10% of the Esil and Ecro haploid and diploid subpopulations harboured samples that consisted only of unique multilocus genotypes (i.e. the sub-populations Gandario for Esil and Plymouth for Ecro, over the 22 studied in Table 4), all the other sub-populations showed repeated multilocus genotypes. Clonal diversity (R values, Table 4) ranged from 0.37 to 1 for Esil and from 0.55 to 1 for Ecro, suggesting that clonal propagation might be important in some sites. F_{IS} values were either not significantly different from zero or

significantly positive depending on the species and populations (Table 4). All but one Ecro population exhibited significantly high heterozygote deficiencies, while most Esil populations (6 over 9, Table 4) did not show any significant departure from random mating. Differentiation among populations (global F_{ST} value, Weir & Cockerham, 1984) calculated for each species and each haploid and diploid subpopulation was significant (Esil: F_{ST} diploid sporophytes = 0.15, F_{ST} haploid gametophytes = 0.45; Ecro: F_{ST} diploid sporophytes = 0.11, F_{ST} haploid gametophytes = 0.08).

Discussion

Species-diagnostic markers to detect hybridization in cryptic species

The lack of any obvious phenotypic divergence between *Ectocarpus* species of the *siliculosi* group has made the study of hybridization in the field a challenge (but see Peters *et al.* 2010a; Couceiro *et al.* 2015 and Montecinos *et al.* in press) since neither the cryptic parental species nor their intermediate hybrid forms can be distinguished morphologically in the field or even in the laboratory. The use of the cytoplasmic species-specific marker rps14-atp8 and the nuclear marker ITS1 in combination with 9 microsatellite loci (6 being diagnostic between species and 3 showing diagnostic alleles), however, allowed us to assign individuals to species and to detect signals of genetic admixture between *E. siliculosus* and *E. crouaniorum*. Both the admixture and assignment analyses demonstrated a high discriminatory power of the microsatellite species-diagnostic markers, at least in as far as distinguishing parental from admixed individuals was concerned. The Bayesian method we developed produced results that were congruent with the classical STRUCTURE approach (e.g. Ito et al. 2015; Turchetto et al. 2015) but the former has the advantage that it mathematically formalizes and automates the computation of the posterior probabilities of individuals to distinct scenarios of hybridization. As secondary contacts are often associated with changes in ploidy that either affect the entire genome or only some chromosomes (aneuploidy) (Livingstone & Rieseberg 2004; Soltis & Soltis 2009), our method can be applied to raw genetic data without correcting them for ploidy. Although our method still

requires the provision of the genotypes of the individuals identified as belonging exclusively (as far as possible) to the parental species involved, it can assign individuals to hybrid categories without clustering them into populations and thus does not rely on biological assumptions such as Hardy-Weinberg equilibrium, sexual reproduction or maximal divergence between populations. Indeed, a large number of true hybrids were identified with XPLOIDASSIGNMENT and, in particular, the method allowed the identification of hybrids in the haploid subpopulation. Only half of the hybrids could be assigned to a single hybrid class with confidence but the distribution of posterior probabilities between admixture scenarios helped to reconstruct more complex hybridization histories.

The species-specific ITS1 marker was generally consistent with the species-diagnostic microsatellite markers and all the individuals bearing both ITS1 lengths were identified as admixed. However, of the 79 admixed individuals revealed by our study, eight presented only the ITS1 band specific of *E. siliculosus* and 12 presented only the ITS1 band specific of *E. crouaniorum* (Table S1b, Supporting information). Concerted evolution (Arnheim *et al.* 1980) could possibly explain why these hybrid individuals lost the ITS1 sequence of one parental species. This process causes the homogenization of tandem repeat sequences through genomic mechanisms such as gene conversion and unequal crossing over (Dover 1994) and has been invoked as the mechanism responsible for ITS homogenization after hybridization events in plants. Five hybrids identified as F1 in our study showed only one parental ITS1 band, which suggests that the process of concerted evolution may happen in the first hybrid generation. In marine seaweeds, intraspecific and intra-individual polymorphisms of ITS are well documented and have been related presumably to incomplete homogenization under concerted evolution after events of recent speciation, hybridization, shifts to asexual reproduction or polyploidization (Pillmann *et al.* 1997; Serrão *et al.* 1999; Famá *et al.* 2000; Coyer *et al.* 2001). Our results indicate that barcoding methods based on single markers should be used with caution.

A low level of hybridization and potential reproductive barriers

The frequency of hybridization between *E. siliculosus* and *E. crouaniorum* was estimated to be 8.7% based on the whole data set (haploid and diploid individuals). Hybrids showed positive amplification of the cytoplasmic marker of both species suggesting that inter-specific crosses occurred in both directions. Hybridization was only detected in sympatric/parapatric populations and hybrids were almost exclusively diploid sporophytes. In addition, most hybrids seemed to have originated recently (i.e. to correspond to the first hybrid generation). Together these results suggest that postzygotic mechanisms limit sexual fertility in admixed individuals. Indeed, the near absence of admixture in haploid subpopulations suggests that meiosis probably acts as a strong post-zygotic barrier in the first hybrid generation (Fig. 1). However, the detection of a small number of hybrids that correspond to subsequent generations of backcrosses in haploid and diploid hybrids on the one hand and the occurrence of aneuploidy on the other, suggest that the reproductive barrier, although strong, is probably not absolute.

Along the shore, adjacent populations of different species and their hybrids have been shown to segregate along environmental gradients (e.g. tide level or difference zones of wave exposure) parental types being favoured at the opposite ends of the ecotone and hybrids occurring in between (see for example in marine invertebrates: Johannesson 2009; Bierne *et al.* 2003). Among seaweeds, the best-studied examples of hybridization come from various species of the genus *Fucus,* which dominate the intertidal rocky shores of the North Atlantic (Coyer *et al.* 2002; Wallace *et al.* 2004; Billard *et al.* 2005; Engel et al. 2005; Coyer *et al.* 2007; Neiva *et al.* 2010; Coyer *et al.* 2011, Monteiro *et al.* 2012). The occurrence of hybridization between these species was first explained by their recent and rapid radiation within the last 3.8 million years, which resulted in a complex of highly related sister species (Serrão *et al.* 1999; Coyer *et al.* 2006). In addition, habitat-driven speciation has been invoked to explain divergence despite the occurrence of gene flow in this genus (in *F. vesiculosus/F. radicans*: Bergström *et al.* 2005;

Pereyra *et al.* 2009; and in *F. spiralis/F. vesiculosus/ F. guyrii*: Zardi *et al.* 2011). Indeed, the different species are often found in sympatry/parapatry along the European coast but distributed along the vertical selective gradient of the intertidal zone (i.e. in different abiotic and biotic micro-environmental habitats). In such a complex context, Hoarau *et al.* (2015) showed that the level of hybridization and interspecific fertilization success decreased with increasing time of sympatry between *F. serratus* and *F. distichus* suggesting reinforcement of isolation mechanisms.

In filamentous Ectocarpales, the lack of obvious phenotypic divergences between cryptic species (see Peters *et al.* 2015) explains why relatively little is known about their distribution, the importance of species co-occurrence and their level of hybridization in the wild. However, thanks to the development of species-specific molecular markers, recent studies have reported that cryptic species may be distinguished on the basis of their spatio-temporal ecological niches related to different tide levels and/or host specificity (in *Ectocarpus spp.*, see references below; in *Pylaiella* spp., Christophe Destombe com. pers.). In particular, Peters *et al.* (2010a), Couceiro *et al.* (2015) and Montecinos *et al.* (in press) showed that *E. crouaniorum* was located higher on the shore than *E. siliculosus* at several sites of the North Atlantic. In addition, in a detailed spatio-temporal study of a North-Western French population, Couceiro *et al.* (2015), reported differences in host specificity between these two species with *E. crouaniorum* being exclusively found on a single species while *E. siliculosus* occurred on more than five species. However, all three studies mentioned that along the shore gradient the two cryptic species could co-occur in a contact zone where their distributions overlap. Moreover, Couceiro *et al.* (2015) reported that the two species could be found on the same host, since *E. siliculosus* was able to grow on *Scytosiphon lomentaria*, the only host species for *E. crouaniorum.* This situation resembles that of the *Fucus* hybridization zone along the steep gradient of the shore. At sites where the two *Ectocarpus* species are found in sympatry, ecological barriers such as habitat preference

combined with host specificity may limit hybridization. In addition, the high level of inbreeding observed in Ecro reveals that mating is not random in this species and this may also contribute to reducing hybridization.

Rarity of haploid hybrids and the existence of post-zygotic reproductive isolation

Unlike *Fucus* species, which have a diploid life cycle, the distinguishing feature of the haploid-diploid life cycle of *Ectocarpus* species makes it possible to distinguish the results of fertilization and meiosis by comparing diploid and haploid sub-populations. In this study, we observed very few recombinant haploid individuals suggesting the presence of strong post-zygotic barriers, which confirmed previous results from crossing experiments between *E. crouaniorum* and *E. siliculosus* (Peters et al. 2010a), in which most hybrid zygotes did not develop beyond an early germination stage. Moreover, the presence of abortive unilocular sporangia (i.e. the site where spores are formed by meiosis on a diploid sporophyte) was observed on hybrids derived from crosses between *Ectocarpus* strains from geographically distant origins (Müller 1988; Stache 1990). Müller (1988) attributed the abortive unilocular sporangia to severe problems in chromosome pairing probably linked to differences in genome size (Peters *et al.* 2004a). Interestingly, in this study, we observed that 40% of hybrid diploid sporophytes possessed three alleles for at least one microsatellite locus. In all these cases, heterozygotes with 3 alleles were composed of two alleles diagnostic of one species with the third allele diagnostic of the second species. These triallelic heterozygotes might reflect the occurrence of aneuploid gametes. Such patterns of abnormal segregation during meiosis could be a consequence of karyotypic rearrangements in chromosomes between *E. siliculosus* and *E. crouaniorum* creating chromatids with duplications and deficiencies in gene content (recombination suppressors as described in the review of Livingstone & Rieseberg 2004). Such karyotypic rearrangements could explain the very low frequency of recombinant hybrid haploids detected in our study. In plant species, reproductive isolation has been largely linked to translocations that rearrange the genome of one species relative to another (Stebbins 1971, Quillet *et al.* 1995, Spirito 1998,

Rieseberg 2001). Moreover, in angiosperms, hybrid fertility is often restored by experimentally inducing tetraploidy, suggesting that meiotic recombination between different karyotypes led to F1 hybrid sterility (Stebbins 1958). Such chromosomal rearrangements have been linked to reduced fertility in hybrids produced in haploid-diploid species (e. g. mosses: Anderson & Snider 1982, yeasts: Liti *et al.* 2006, ferns: Wagner 1987). However, whether such rearrangements contribute to the speciation process is a matter of debate (Sites & Moritz 1987; Coyne & Orr 2004). We observed that some of the identified diploid sporophyte hybrids shared the same multi-locus genotypes suggesting the occurrence of clonal multiplication of these hybrids. However, as asexual reproduction also occurs in the parental populations, it is difficult to ascertain whether the observed clonal multiplication of diploid sporophytes is favoured because of post-zygotic barriers. Finally, despite the extreme rarity of recombinant genotypes in the haploid dataset, analyses of simulated backcrosses suggested the occurrence of subsequent generations after F1 sporophyte hybrids in our dataset. This result suggests that post-zygotic incompatibility might not be absolute between both species. However, several studies have argued that identifying individuals with hybrid ancestry could become increasingly difficult after the first three generations using the methods we employed in this paper (e.g. Lavrestky *et al.* 2016). Thus we cannot reject the hypothesis that these later hybrid generations were misidentified. In order to verify the importance of post-zygotic isolation, several further experiments can be suggested. First, as all genotyped individuals are maintained as uni-algal cultures in the lab, the occurrence of meiosis can be tested experimentally for each of the 77 identified hybrid sporophyte strains. Second, using next-generation sequencing, it is now possible to examine genome-wide patterns of introgression and identify genomic regions that show signatures of selection.

Presence of rare alleles in hybrids.

The rare allele phenomenon refers to the observation that certain alleles, which are normally rare or virtually non-existent in both parental species, can occur at high frequencies in the

centre of the hybrid zone. The rare allele phenomenon has been observed in hybrid zones of land plants and animals using a wide range of markers, including intron haplotypes (Schilthuizen *et al.* 1999), rDNA spacer variants (Liao *et al.* 2010), microsatellites (Lexer *et al.* 2007) and SNPs (Lammers *et al.* 2013). Coyne & Orr (2004) have hypothesized that, in the centre of a tension zone, the continuous generation of low-fitness recombinants will favour any allele that decreases hybrid disadvantage. Our results revealed that all 96 rare alleles were present in hybrids and two displayed a considerably higher frequency in hybrids compared to parental species. This observation may explain some of the discrepancies between the three assignment methods used to detect hybridization (i.e. hybrid genotypes where many rare alleles are encountered could be difficult to assign to the simulated hybrid classes since only the pure bred parental species were used to generate the simulated hybrid genotypes in GENECLASS or XPLOIDASSIGNMENT). In this study with *Ectocarpus* species, it remains unclear if the zone in which the two species are in contact in sympatric populations can be considered to be a tension zone. Whether or not the presence of rare alleles in hybrids is a product of the rare allele phenomena or misestimation of allele frequencies will require further sampling specifically designed to address this question.

Conclusion

Studies of hybridization in haploid-diploid species can be particularly rewarding, as it is possible to separate pre-zygotic from post-zygotic barriers, which is not easily done in species lacking such life cycles. The diversity of taxa with various levels of divergence in *Ectocarpus* (Stache-Crain *et al.* 1997; Peters *et al.* 2010b; Peters *et al.* 2015, Montecinos *et al.* in press) makes this genus a good model for studies of reproductive isolation. Crossing experiments between *E. siliculosus* and the genetically more distant species *E. fasciculatus* have shown that plasmogamy was only possible between male gametes of *E. fasciculatus* and females of *E. siliculosus*, however the hybrids died after germination (Müller & Gassmann 1980). In contrast,

cytoplasmic introgression among closely related species belonging to the *E. siliculosi* group was suggested by Montecinos *et al.* (in press). Together, these results suggest a probable relationship between genetic distance and cross-compatibility in this genus. Follow-up studies on this system could provide further insights into the evolutionary consequences of hybridization and introgression for the maintenance or breakdown of species.

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Data Accessibility

Parental microsatellite genotypes uploaded in Dryad doi:10.5061/dryad.43hd6.

Author Contributions

M.L.G. and M.V. conceived and designed the study. A.E.M., A.F.P., L.C. and M.V. collected samples. A.E.M., A.F.P. and L.C. established unialgal cultures. A.E.M. and L.C. genotyped and barcoded the samples. A.E.M., M.L.G., and M.V. performed data analysis and synthesis. S.S. developed the new method and software XPLOIDASSIGNMENT. A.E.M. wrote the manuscript with contributions from all authors.

Figure captions

Figure 1. The haploid-diploid life cycle model to study hybridization and reproductive barriers.

In diploid life cycles, mitotic cell division and somatic development occur entirely in the diploid phase. The haploid stage is reduced to a single-cell (i.e. gamete) produced through meiosis in the diploid phase and fertilization occurs immediately after release of gametes to recreate the diploid phase. Therefore, population genetics studies based on diploid life cycles are focused only on diploid individuals. On the other hand, in haploid-diploid life cycles, somatic development occurs in both haploid and diploid phases and there is an alternation between two types of independent functional individuals: haploid gametophytes produced by meiosis and diploid sporophytes resulting from fertilization. Haploid diploid life cycles provide a unique opportunity to estimate the frequency of hybrid genotypes in diploid sporophytes and meiotic recombinant genotypes in haploid gametophytes allowing the effects of reproductive barriers that prevent fertilization to be distinguished from those that prevent meiosis.

Figure 2. Principal component analysis of *E. siliculosus*, *E. crouaniorum* and putative hybrid individuals presented for a) all diploid sporophytes and b) all haploid gametophytes using the nine microsatellite loci. First and second axes represent the first two factorial components.

The legend of the different symbols refers to the two parental species *E. siliculosus* (Esil) and *E. crouaniorum* (Ecro) and the four types of putative of hybrid (2ITS1 + mt-Esil: presence of the two ITS1 species-specific bands and mitochondrial specific marker of Esil; 2ITS1 + mt-Ecro: presence of the two ITS1 species-specific bands and mitochondrial specific marker of Ecro; ITS1 Esil + mt-Esil: presence of the ITS1 band specific of Esil and mitochondrial specific marker of Esil; ITS1 Ecro + mt-Ecro: presence of the ITS1 band specific of Ecro and mitochondrial specific marker of Ecro). The number of diploid sporophytes (SP) and haploid gametophytes (GA) observed in each putative hybrid category is given in Table S1a (Supporting information).

Figure 3. Clustering analysis performed with STRUCTURE using the nine microsatellite loci. Results are shown for K2 for a) all diploid sporophytes and b) all haploid gametophytes. Each vertical bar represents a different individual. The shading represents the proportion of individual genome assigned to each genetic group (grey *E. siliculosus* and white *E. crouaniorum*). Sampling sites are noted below and sites are ordered from North to South.

Figure 4. Principal component analysis of all observed diploid sporophytes compared with the three classes of hybrid simulated using ADEGENET HYBRIDIZE (Jombart 2008). The parental species *E. siliculosus* and *E. crouaniorum* and the 79 putative hybrids are represented as black squares, grey triangles and red circles, respectively. The 500 simulated genotypes of each hybrid class are represented as circles: F1 (green), backcrosses with *E. siliculosus* (blue) and backcrosses with *E. crouaniorum* (purple). First and second axes represent the first two factorial components.

Table 1. Linkage disequilibrium among molecular markers used for species identification: *rps*14-*atp*8 spacer (mtDNA) and the ITS1 (nrDNA) for diploid sporophytes (*SP*) and haploid gametophytes (*GA*) identified using the sex-specific markers.

ITS1	**Ploidy/phase**	**mt-*Esil***	**mt-*Ecro***	**Total**
Esil	*SP*	347	0	347
	GA	158	0	158
	Total SP+GA	505	0	505
Ecro	*SP*	0	162	162
	GA	0	178	178
	Total SP+GA	0	340	340
Esil+Ecro	*SP*	14*	45*	59*
	GA	0	0	0
	Total SP+GA	14*	45*	59*
Total	***SP***			**568**
	GA			**336**
	Total SP+GA			**904**

Esil = *E. siliculosus*, *Ecro* = *E. crouaniorum*, *Esil+Ecro*: individuals bearing both ITS1 bands, *: putative hybrids

Table 2. Linkage disequilibrium among the mitochondrial species-specific markers (*rps*14-*atp*8 spacer) and the nine nuclear microsatellite loci. The table shows the frequency of amplification of each locus for the two groups of individuals (mt-Esil and mt-Ecro) identified based on the cytoplasmic information. Microsatellite loci were classified as diagnostic for *E. siliculosus*, diagnostic for *E. crouaniorum* or showing diagnostic alleles. The frequency of amplification and allele size were used to classify the nine microsatellite loci.

Locus category	Locus name	**mt-*Esil* (N=519)**	**mt-*Ecro* (N=385)**
		Amplified	Amplified
Diagnostic loci *Esil*	M-033-1	489	51*
	M-239-3	501	48*
	M-103-2	513	47*
	M-387	508	48*
Diagostic loci *Ecro*	M-388	23*	374
	M-420	14*	338
Diagnostic alleles	M-122-2	518	376
	M-208	515	384
	M-162-1	513	385

mt-*Esil*: cytoplasmic identification as *E. siliculosus*, mt-*Ecro*: cytoplasmic identification as *E. crouaniorum*, diagnostic loci: loci that amplify only or mostly in one cytoplasmic group, diagnostic alleles = loci that amplify in both cytoplasmic groups but but for which alleles differ in size between mt-*Esil* and mt-*Ecro* (see Fig. S2, Supporting information).

* putative hybrid individuals showing amplification of the species-specific loci of both species.

Table 3. Number of diploid sporophytes and haploid gametophytes collected at each site and their identification as parental species or putative hybrids. Hybrids were identified using a combination of three methods: XPLOIDASSIGNMENT, GENECLASS and STRUCTURE.

		Esil		***Ecro***		**Hybrids[$]**			**Frequency of hybrids**
Regions	**Populations**	**SP**	**GA**	**SP**	**GA**	**SP**	**GA**	**SP+GA**	
U.K.	Plymouth*	44	1	0	18	1	0	1	0.015
	Restronguet	30	12	0	0	0	0	0	0.000
Brittany	Roscoff*	124	4	62	50	14	0	14	0.055
	Le Caro	13	4	0	0	0	0	0	0.000
	Traezh Hir*	33	8	4	57	14	0	14	0.121
	Quiberon*	25	1	23	26	1	0	1	0.013
NW Iberia	Ribadeo*	16	0	18	23	18	0	18	0.240
	Gandario*	22	0	1	0	7 (8)	0	7 (8)	0.133
	Lourido*	4	0	44	2	22 (23)	2	24 (25)	0.333
Mediterranean	Naples	28	128	0	0	0	0	0	0.000
	TOTAL	**339**	**158**	**152**	**176**	**77 (79)**	**2**	**79 (81)**	

**sympatric populations*

[$]: The two putative hybrids consistently classified as parental species by all methods are shown within brackets

Esil = *E. siliculosus*, *Ecro* = *E. crouaniorum*, SP= diploid sporophytes, GA= haploid gametophytes

Table 4. Genotypic diversity and deviation from random mating in *E. siliculosus* (7 loci) and *E. crouaniorum* (5 loci).

Species	Ploidy	Populations	N	MLG	R	F_{IS}	
E. siliculosus	2n	Plymouth	44	17	0.37	0.023	NS
		Restronguet	30	23	0.76	-0.045	NS
		Roscoff	104	65	0.62	0.134	***
		Traezh Hir	27	13	0.46	0.095	*
		Le Caro	11	10	0.90	-0.056	NS
		Quiberon	25	15	0.58	-0.003	NS
		Gandario	19	19	1.00	0.093	*
		Ribadeo	15	12	0.79	0.076	NS
		Naples	26	17	0.64	0.047	NS
	n	Restronguet	12	11	0.91	---	
		Traezh Hir	7	5	0.67	---	
		Naples	117	47	0.40	---	
E. crouaniorum	2n	Roscoff	54	51	0.94	0.176	***
		Quiberon	21	12	0.55	-0.024	NS
		Lourido	45	34	0.75	0.229	***
		Ribadeo	15	14	0.93	0.255	***
	n	Plymouth	12	12	1.00	---	
		Roscoff	43	41	0.95	---	
		Quiberon	18	16	0.88	---	
		Ribadeo	19	18	0.94	---	
		Traezh Hir	37	34	0.92	---	

N= number of individuals genotyped, MLG= number of different multilocus genotypes, R= clonal diversity, F_{IS}= inbreeding coefficient and test for deviation from random mating: NS: non significant, *: $p<0.05$, ***:$p<0.01$.