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Sex or Fission? Genetics Highlight Differences in Reproductive Strategies of Two Sympatric Fissiparous Sea Cucumber Species in Reunion Island (Southwestern Indian Ocean)

Joséphine Pierrat [1,*], Nicolas Oury [1,2], Patrick Frouin [1,3] and Hélène Magalon [1,2,3]

1 UMR ENTROPIE (Université de La Réunion, Université de Nouvelle-Calédonie, IRD, CNRS, IFREMER), Faculté des Sciences et Technologies, Université de La Réunion, 15 Bd René Cassin, CS 92003, 97744 St. Denis, CEDEX 09, La Réunion, France; nicolasoury@hotmail.fr (N.O.); patrick.frouin@univ-reunion.fr (P.F.); helene.magalon@univ-reunion.fr (H.M.)
2 Laboratoire Cogitamus, 75000 Paris, France
3 Laboratoire d'Excellence Corail, 66100 Perpignan, France
* Correspondence: josephine.pierrat@univ-reunion.fr

Abstract: *Holothuria leucospilota* and *Stichopus chloronotus* are among the most widespread tropical sea cucumber species usually harvested for food and medicine in Asian countries, for which natural stocks have collapsed worldwide. Both species can reproduce sexually and asexually, and a better understanding of their reproductive strategy can provide useful information for conservation purposes. To describe the genetic structure and diversity of sympatric populations from these species in space and time, individuals were sampled over different sites and seasons in Reunion Island (Southwestern Indian Ocean). They were genotyped using 24 and 9 specific microsatellite markers for *H. leucospilota* and *S. chloronotus*, respectively. Multi-locus genotypes (MLG) and lineages (MLL) were identified, and analyses of population structure were performed among sites and seasons. No repeated MLG nor MLL were found for *H. leucospilota*, demonstrating the absence of asexual reproduction. Populations of *H. leucospilota* were not genetically differentiated, acting as a metapopulation, with larval exchanges within the reef. Contrarily, repeated MLGs were found for *S. chloronotus* and all populations were genetically differentiated. Asexual reproduction seems to reach a high level for this species (mean clonal richness = 0.24). For both species, genetic structure was stable through seasons. Thus, these sympatric fissiparous sea cucumber species use two different strategies of reproduction, which may allow them to reduce interspecific competition.

Keywords: *Holothuria leucospilota*; *Stichopus chloronotus*; sea cucumber; microsatellite; genetic structure; clonal propagation; reproductive strategies; sympatric species; interspecific competition

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1. Introduction

Sea cucumbers are among the most abundant benthic megafauna species in many ecosystems, such as the deep-sea [1,2], corals reefs [3] and shallow marine habitats [4]. Among the 1750 species currently described [5], approximatively 70 species are harvested [6] for food (commonly known as "bêche-de-mer" or "trepang"), traditional medicine and aphrodisiacs for many Asian countries [7–9]. Only two species present a complete process of domestication for large-scale aquaculture purposes, from egg spawning to broodstock maintaining: *Holothuria scabra* [10] and *Apostichopus japonicus* [11]. During the last decades, fisheries of sea cucumbers have quadrupled [12] and their coastal populations have been decimated by hand collecting [13] to satisfy the increasing demand of the Asian market [14]. The depletion of stocks of high-commercial value species has led to a shift toward low-commercial value species [15]. Fishing regulations and management plans are insufficient to restore some local populations [13]. Therefore, data on demographical parameters and genetic structure of sea cucumber populations are needed to establish efficient

management plans, to increase the number of species for aquaculture and, consequently, to avoid the depletion of natural stocks and the loss of ecosystem services provided by sea cucumbers.

Holothuria (Mertensiothuria) leucospilota (Brandt, 1835) and *Stichopus chloronotus* (Brandt, 1835) are on the original FAO list of commercial species [15]. *Holothuria leucospilota*, commonly called "black long sea cucumber" or "white thread fish", is one of the most widespread sea cucumber species, inhabiting the Western Central Pacific, Asia and Indian Ocean and living on soft substrates in coral reefs and shallow coastal habitats [15]. *Stichopus chloronotus*, called "greenfish", is also largely distributed throughout the Indo-West Pacific, living on coarse corals and coral rubbles [15]. Dried body wall of *H. leucospilota*, considered as low-commercial value species, can be sold up to 5 USD·kg^{-1} in the Philippines [15], whereas up to 95 USD·kg^{-1} for *S. chloronotus*, considered as medium-commercial value species [16]. Individuals are harvested by hand collecting at low tide, mainly in Madagascar and many Asian countries, where low and medium-value species are fished without any restriction. Moreover *S. chloronotus* is exploited in artisanal and semi-industrial fisheries, mostly in Mauritius [15]. Gonads of *H. leucospilota* are traditional subsistence in Cook Island culture [17], and active substances have been isolated from the body wall for medicine applications, such as antibacterial and antifungal [18], antioxidant [19,20] and antitumoral [21] activities. *Stichopus chloronotus* is harvested for subsistence consumption in some islands and is commercially important for food in many Asian countries [15]. Increasing knowledge on the genetic structure and diversity of these two species would allow to better understand their ecology and to preserve natural stocks from depletion by helping their domestication for aquaculture purposes.

In contrast with some certain localities where they are highly harvested, *H. leucospilota* and *S. chloronotus* are distinguished by their exceptional densities in Reunion Island (Southwestern Indian Ocean), which homes 38 species of sea cucumber [22]. Populations of *H. leucospilota* and *S. chloronotus* are found in sympatry in the west and south coasts of Reunion Island, mainly in the fringing reef of L'Hermitage/La Saline and Étang-Salé. They are monitored since 25 years and the observed densities ranged between 0.15 and 3.7 ind·m^{-2} depending on the location [23–26]. These species are among the 16 species of sea cucumber having the ability to reproduce both sexually through gamete spawning and asexually by transversal fission [27]. Sexual reproduction leads to the first larval stage (auricularia), which feeds on phytoplankton whereas, in asexual reproduction, one individual undergoes fission, leading to two deposit-feeder adults. The fission rates for populations of *H. leucospilota*, estimated thanks to a visual census of individuals undergoing fission, ranges between 5% in Reunion Island [23] and 28% in Australia [28]. Although *H. leucospilota* is one of the most common sea cucumber species, only two studies have investigated its genetic diversity [29,30], and no study has ever evaluated the genetic structure and clonal propagation of its populations using genetic tools. The number of individuals of *S. chloronotus* performing fission has been estimated to 17% at Reunion Island [24], using the same method as for *H. leucospilota* [23]. However, two decades later, the clonal richness was analysed using nine microsatellite markers [31] and authors concluded that it was extremely low (R = 0.09), meaning that many individuals of *S. chloronotus* are clones, and therefore, have participated or participate to asexual reproduction. Visual census is not a good predictor to estimate clonal propagation, as concluded by a study on *Holothuria atra* [32]. Genetic analyses thus need to be realised on *H. leucospilota* to evaluate the importance of asexual reproduction in the populations of Reunion Island.

Here, we focused on the populations of *H. leucospilota* and *S. chloronotus* from Reunion Island, collected at different sites and dates, to (1) identify clones to estimate the level of asexual propagation, (2) estimate the genetic diversity of these populations and (3) estimate the genetic structure among populations of each species, to investigate a potential genetic connectivity among reefs and seasons, and the impact of the two reproductive strategies through time.

2. Materials and Methods

2.1. Sampling Design

Sampling was carried out on the west coast of Reunion Island (Southwestern Indian Ocean; 21°06′ S, 55°31′ E), in the fringing reefs of L'Hermitage/La Saline and Étang-Salé (Figure 1). Individuals of *H. leucospilota* and *S. chloronotus* were haphazardly sampled by hand collecting in the back-reef depression and stored at −80 °C before analyses.

2.1.1. Spatial Sampling

Individuals of *H. leucospilota* were sampled only along the reef of L'Hermitage/La Saline because none was found in the reef of Étang-Salé. Three sites with a high density (>1 ind·m^{-2}, Ref. [25] and J.P. Pers. Comm.) were chosen: MNS (Maître-Nageur-Sauveteur, 2.6 ± 0.2 ind·m^{-2}), PLA (Planch'Alizé, 1.0 ± 0.1 ind·m^{-2}) and TE (Trou d'Eau, 1.2 ± 0.1 ind·m^{-2}) (Figure 1). Two additional sites with low densities (<0.1 ind·m^{-2}, personal observations) were studied: CAP (Cap Méchant) and PTE (Petit Trou d'Eau) (Figure 1). Individuals of *S. chloronotus* were sampled in the same high density sites as in a previous study [31]: PAS (Passe de l'Hermitage; corresponding to HIGH1 in [31]; with a density of 0.8 ± 0.1 ind·m^{-2}), TE (Trou d'Eau; HIGH2; 1.2 ± 0.1 ind·m^{-2}), both in the reef of L'Hermitage/La Saline, and ES (Étang-Salé; HIGH3; 0.7 ± 0.1 ind·m^{-2}) in the reef of Étang-Salé.

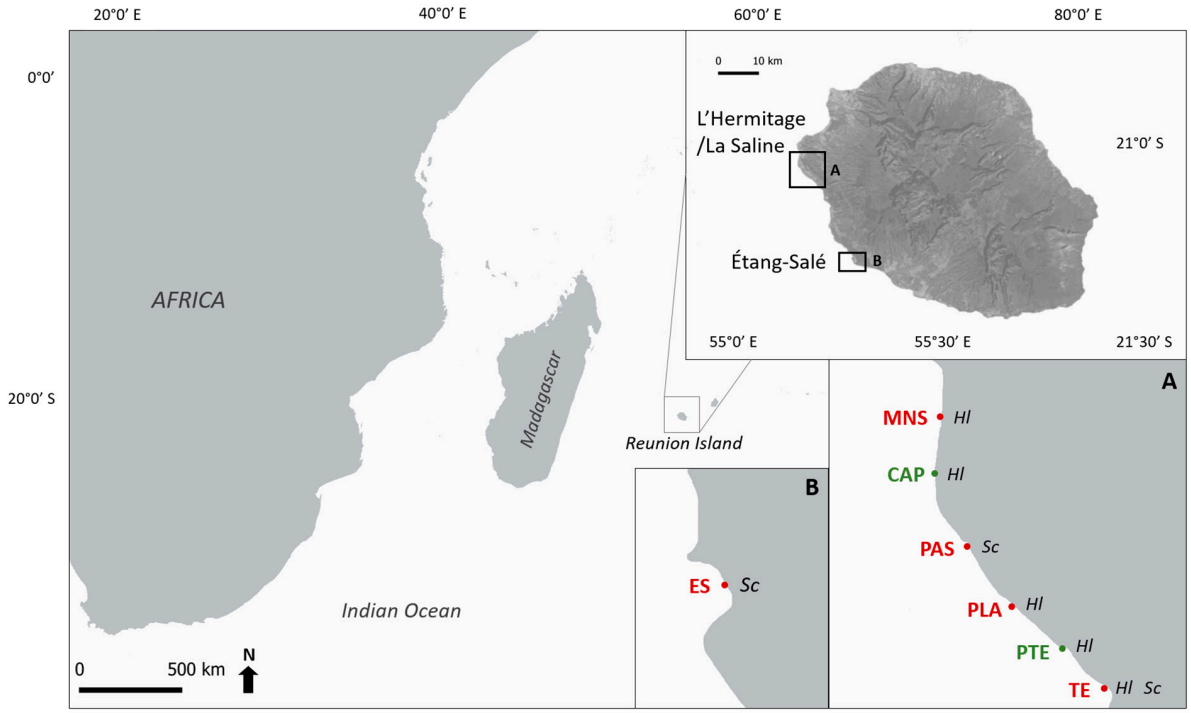

Figure 1. Location of the sampling sites of each reef. High density sites in red: MNS: Maître-Nageur-Sauveteur, PAS: Passe de l'Hermitage, PLA: Planch'Alizé, TE: Trou d'Eau, ES: Étang-Salé. Low density sites in green: CAP: Cap-Méchant, PTE: Petit Trou d'Eau. *Hl*: *Holothuria leucospilota* and *Sc*: *Stichopus chloronotus* indicate where species were harvested.

2.1.2. Temporal Sampling of Both Species

To analyse the effect of the two strategies of reproduction (sexual and asexual) on the genetic structure, sampling was performed for three consecutive seasons: the cold season 2019 ($S1_{cold}$: austral winter in September 2019), warm season 2020 ($S2_{warm}$: austral summer in February 2020) and cold season 2020 ($S3_{cold}$: austral winter in September 2020). For each site, 24 individuals were sampled, except for low-density sites (CAP and PTE), where only 12 individuals were collected due to the low densities observed. Sampling design for both

species is summarised in Figure S1. For a given species, a population is considered as all the individuals sampled at a given site and a given season.

2.2. Laboratory Steps

Total genomic DNA was extracted from a small piece of tegument, using the DNeasy Blood and Tissue kit (Qiagen™, Hilden, Germany), following the manufacturer's protocol. Individuals of *H. leucospilota* and *S. chloronotus* were genotyped using 24 [33] and 9 [31] specific microsatellite loci, respectively. Forward primers were indirectly fluorochrome labelled (6-FAM, VIC, NED) and were multiplexed post-PCR in panels (Tables 1 and 2, for *H. leucospilota* and *S. chloronotus*, respectively). PCRs were then performed with Veriti™ Thermal Cyclers, in a total volume of 10 µL with MasterMix Applied 1X (Applied Biosystems, Waltham, MA, USA), 0.025 µM of forward primer tagged with the M13 tail, 0.25 µM of reverse primer, 0.25 µM of fluorescent dyed M13 tail and ca. 2 ng·µL^{-1} of genomic DNA. The thermocycling program was the following: 94 °C for 5 min and 7 × (94 °C for 30 s, 62 °C [−1 °C at each cycle] for 30 s, 72 °C for 30 s) and 35 × (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) and 8 × (94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s) and 72 °C for 5 min. PCR products were genotyped using an ABI3730XL sequencer (Applied Biosystems) at the Plateforme Gentyane (INRAE, Clermont-Ferrand, France). Allelic sizes were determined with GeneMapper 4.0 (Applied Biosystems) using an internal size standard (Genescan LIZ-500, Applied Biosystems).

Table 1. Panels for multiplexing the 24 *Holothuria leucospilota* microsatellite loci.

Panel	Locus	Primer Sequence (5′–3′)	Dye	Specific Size Range (bp)
1	Hl21	F: TGTTTCACGAATGAATGAACG R: GCTTGTAAAGCCATTTGTACCTT	6-FAM	220–320
	Hl04	F: CCCAGAAGCTCTGGAACATT R: TGCTATGTAAACTGAAGCCAAA	VIC	170–184
	Hl10	F: AAACGTCCTCGATTGACAGC R: TCTGCTAGCCAAATTACAGGG	NED	137–165
	Hl19	F: GCCGATTCCTTTGAACATTA R: AATTGGTTGGAAACTGGGAC	6-FAM	91–132
2	Hl23	F: GGTCAAAGAACCTGCAGACA R: CCCGACTCAAGCATTACTTAAA	6-FAM	238–274
	Hl06	F: CGTCACGTTACGAATGGTACTC R: TTGGCGCATTTCCTTACAAT	VIC	192–208
	Hl15	F: TCCAAGTATGAGATCCGTCG R: CAGTCCTTGCCGAATGCT	NED	144–168
	Hl08	F: AATCTGGTCTGCTTTCAGGA R: AAACTGCCTGGGTAAGTCTGT	6-FAM	126–138
3	Hl01	F: ATCGTGTTTACAAGCTAGGCG R: AGATGTTGCTAGACCACTGCAT	6-FAM	239–291
	Hl05	F: ATTGGCAGGCAAGGAATCTA R: GTCTATGTCGCCTGATGGCT	VIC	166–180
	Hl03	F: TTTCATTATGTTGCACCCACC R: TGTAAAGCACAACTTTGCGTG	NED	134–156
	Hl14	F: TGCAGTGCCATATCCAACAT R: TTCTTTCATCCTCTCGGCAT	6-FAM	129–149
4	Hl12	F: CAGCACATAGTATACTGCATTCCC R: AAATTCCGTCACTGCAAAGAA	6-FAM	268–278
	Hl16	F: TAGAAATCCTTTCCGCGTGT R: GATGCCCTCGGATTGTATGT	VIC	200–228
	Hl13	F: CAAGTGTTCCAAACTGGGCT R: TCTTCGGGAAGTGTTAGTTGC	NED	133–165
	Hl20	F: CGGGTGCAGAAAGTACCCTA R: GGTTCCAACTCCCTGGTCTT	6-FAM	130–174

Table 1. *Cont.*

Panel	Locus	Primer Sequence (5′–3′)	Dye	Specific Size Range (bp)
5	Hl24	F: GTTAATACGTCAAGTAACGTAGACTGC R: TTCCTTCTTATTTGGCGAGC	6-FAM	294–304
	Hl11	F: GAACTAACAGCCACGATTGG R: CGCATAAACTGTGAAGAAGATCC	VIC	201–215
	Hl22	F: TCAGGTGATTAGTAGCTCAGCAAG R: CCAACTTTGAGAAGGAACGG	6-FAM	143–185
	Hl02	F: CCGTAAGGCATCGAGTGTG R: ACATTCGAGAAGGAAGCTTGA	NED	130–134
6	Hl17	F: GAATCTTATAATCCCTTGGTTCTCA R: TCGATCTAACATATAGAATCGTTGG	6-FAM	273–321
	Hl07	F: AACTGGCTTCAATGACACTACG R: TTGATCGCTTGGTTATTGAGTT	VIC	205–221
	Hl09	F: GAATAATCACAAGTTTGACGGC R: TAATCTTGAGAAGCCGGTGT	NED	145–189
	Hl18	F: CACGAACAGATTTCTTTGTTGTTC R: TGTGGAAGATCACGGGTAAG	6-FAM	132–174

Table 2. Panels for multiplexing the 9 *Stichopus chloronotus* microsatellite loci.

Panel	Locus	Primer Sequence (5′–3′)	Dye	Specific Size Range (bp)
1	Sc10	F: CGCCTCTAATCTCAAATTGTCG R: TGCGGTCTTCCTTGTCTC	6-FAM	142–164
	Sc09	F: CCAATGCTTTGATTCCAGG R: CCAACTTGCACATATTGAG	VIC	200–206
	Sc43	F: CGTGACATACAACTTCCTAGC R: GAGATCACTTAGAGTTACGC	6-FAM	233–239
	Sc01	F: CGGGAAGCATTAAAAGTCGC R: GCGATACGGATCCTTGTGG	VIC	323–326
2	Sc24	F: CGTGGTTAAATTCCTAGGTATAGAG R: CTGGAATAAACCTGATGTAC	6-FAM	148–158
	Sm007	F: CACCGCTTTGAATTTGTAG R: ACTGTAGGCAATGAATGA	VIC	172–176
	Sc29	F: GTAGCCCATAAATCATTG R: GACCAACCCACACAGCAAG	NED	212–218
	Sc33	F: CTGGTTCGGATTCACATAG R: CTACTTACGGTGAAACTTCC	6-FAM	260–266
	Sm014	F: CACGGACAGTGGTCACAAG R: TGAGATAGAGCGTTTACGAG	VIC	355–365

2.3. Data Analyses

2.3.1. Clonal Identification and Propagation

For each species, the occurrence of identical multi-locus genotypes (MLG) was investigated (considering missing data as potentially identical alleles for *H. leucospilota*), with a custom R [34] script. Then, clonal richness *R* [35] was calculated for each population, with the formula $R = \frac{(N_{MLG}-1)}{(N-1)}$, with N_{MLG}, the number of distinct MLGs and *N*, the number of individuals. Finally, using the same custom R script, the occurrence of multi-locus clonal lineages (MLL; i.e., MLGs sharing a certain number of alleles, considered close enough to be part of the same lineage) was also investigated based on the distribution of pairwise differences among MLGs. If MLLs are present in the population, the distribution of pairwise differences must show a clear antimode in the number of alleles shared, corresponding to the threshold from which all MLGs with less allelic differences belong to the same MLL.

Meanwhile, for *H. leucospilota* populations, to be able to compare the numbers of MLGs and the subsequent clonal richnesses with those of *S. chloronotus* ([31] and this study) and *H. atra* [32], 1000 sub-datasets were created by randomly sampling 1000 times 9 out of the 24 loci used. MLGs and clonal richnesses were then calculated for each sub-dataset, considering missing data as potentially identical alleles, thanks to a custom R [34] script.

2.3.2. Genetic Diversity

The number of alleles (*Na*), the number of private alleles (*Np*), the observed and expected heterozygosities (*Ho* and *He*, respectively) and the inbreeding coefficient (F_{IS}) [36] were estimated with FSTAT 2.9.3.2 [37] for each population of *H. leucospilota* and *S. chloronotus*, keeping all individuals as reported in [31] for comparison purposes. Departures from the Hardy–Weinberg equilibrium (HWE) were tested with Genepop 4.7.0 [38,39].

2.3.3. Population Structure and Differentiation

Bayesian clustering analyses were realised with Structure 2.3.4 [40] for both species, keeping only one representative per MLG for each population. Five chains with 2×10^6 generation steps after a burn-in of 2×10^5 were run, assuming admixture and correlated allele frequencies, for *K* varying from 2 to 5. Discriminant Analysis of Principal Components (DAPC) was also performed using the R package adegenet 2.0.0 [41]. Structure and DAPC outputs were summarised and plotted with CLUMPAK [42]. To find the optimal *K* from the Structure outputs, we used the ΔK statistic [43] in CLUMPAK [42]. The Bayesian Information Criterion (BIC) from the DAPC output was estimated in R. For *S. chloronotus*, Structure and DAPC analyses were also realised keeping only one representative per MLG for each site, with all seasons combined.

F_{ST} [44] were calculated between each pair of populations keeping all individuals for both *H. leucospilota* and *S. chloronotus*, and 1000 bootstraps were realised to test whether F_{ST} values were significantly different from zero using Arlequin 3.5.2 [45] and the False Discovery Rate for multiple tests.

3. Results

3.1. MLG and Clone Identification

3.1.1. Clonal Diversity of *Holothuria leucospilota*

On the 288 individuals genotyped for *H. leucospilota*, 47 did not amplify with at least 10 markers (42 from $S1_{cold}$, 2 from $S2_{warm}$ and 3 from $S3_{cold}$), thus, we decided to remove them from the rest of the analyses. Only 74 individuals (25%) presented MLGs without missing data over the 241 remaining individuals, but none of these MLGs were shared among individuals (clonal richness $R = 1$). The analysis keeping missing data as potential identical alleles (i.e., over-estimating the presence of clones) showed that all individuals have their own MLG (Table 3); therefore, no shared clone was present in the populations of *H. leucospilota*. Clonal richness reached 1 whichever the site density (low or high) or the season (cold or warm) (Table 3). Moreover, no clear antimode was found on the distribution of pairwise differences among MLGs (Table S1), meaning that each MLG is too distant from the others and constitutes a distinct MLL on its own.

The absence of repeated MLG and MLL in the populations of *H. leucospilota* may be due to the high number of microsatellite markers used (i.e., 24 markers), decreasing the probability to find two identical MLGs over the 48 alleles identified. Random selection (1000 sub-datasets) of 9 microsatellite markers, over the 24 used for genotyping individuals, revealed that the mean clonal richness reached 0.99 ($\pm 5.8 \times 10^{-6}$) ($\pm$SE; min: 0.996; max: 1) and that the mean number of MLGs identified was 240.99 (over 241 individuals; min: 240; max: 241), confirming the absence of repeated MLG in the populations of *H. leucospilota* from Reunion Island.

Table 3. Indices of genetic diversity for *Holothuria leucospilota* populations from Reunion Island.

Season	Site	%*NA*	*N*	N_{MLG}	*R*	*Na*	*Np*	*Ho*	*He*	F_{IS}
S1	MNS	58.33	10	10	1	6.17 ± 0.58	0.96 ± 0.24	0.39 ± 0.05	0.82 ± 0.03	0.53 *** ± 0.05
	CAP	50.00	6	6	1	4.96 ± 0.39	0.50 ± 0.17	0.41 ± 0.06	0.82 ± 0.03	0.48 *** ± 0.07
	PLA	37.50	15	15	1	7.04 ± 0.57	1.08 ± 0.21	0.41 ± 0.04	0.80 ± 0.03	0.50 *** ± 0.04
	PTE	41.67	7	7	1	5.58 ± 0.49	0.46 ± 0.16	0.45 ± 0.06	0.80 ± 0.04	0.43 *** ± 0.07
	TE	33.33	16	16	1	7.38 ± 0.65	0.92 ± 0.18	0.33 ± 0.04	0.80 ± 0.03	0.58 *** ± 0.04
S2	MNS	4.17	23	23	1	10.38 ± 0.80	0.96 ± 0.20	0.57 ± 0.04	0.82 ± 0.02	0.31 *** ± 0.04
	CAP	0.00	12	12	1	7.67 ± 0.64	0.42 ± 0.18	0.54 ± 0.05	0.79 ± 0.03	0.31 *** ± 0.05
	PLA	0.00	24	24	1	10.46 ± 0.82	0.75 ± 0.24	0.52 ± 0.04	0.82 ± 0.02	0.37 *** ± 0.04
	PTE	0.00	12	12	1	8.08 ± 0.54	0.50 ± 0.16	0.55 ± 0.05	0.83 ± 0.02	0.34 *** ± 0.05
	TE	4.17	23	23	1	10.17 ± 0.87	0.63 ± 0.19	0.53 ± 0.04	0.80 ± 0.003	0.34 *** ± 0.04
S3	MNS	0.00	24	24	1	10.46 ± 0.70	1.13 ± 0.23	0.53 ± 0.05	0.83 ± 0.02	0.37 *** ± 0.05
	CAP	0.00	12	12	1	7.67 ± 0.49	0.33 ± 0.10	0.50 ± 0.05	0.82 ± 0.02	0.38 *** ± 0.06
	PLA	12.50	21	21	1	9.71 ± 0.62	0.58 ± 0.15	0.59 ± 0.04	0.81 ± 0.02	0.27 *** ± 0.04
	PTE	0.00	12	12	1	7.58 ± 0.58	0.29 ± 0.11	0.52 ± 0.05	0.81 ± 0.03	0.35 *** ± 0.05
	TE	0.00	24	24	1	10.54 ± 0.72	1.38 ± 0.26	0.53 ± 0.04	0.83 ± 0.03	0.36 *** ± 0.04

%*NA*: percentage of missing data; *N*: number of individuals that amplified for at least with 10 markers; N_{MLG}: number of distinct multi-locus genotypes; *R*: clonal richness; *Na*: mean number of alleles; *Np*: mean number of private alleles; *Ho* and *He*: observed and expected heterozygosities, respectively; F_{IS}: inbreeding coefficient and significant deviation from Hardy–Weinberg Equilibrium (***: $p < 0.001$). Standard errors are indicated following mean values. Grey lines represent low density sites. High density sites: MNS: Maître-Nageur-Sauveteur, PLA: Planch'Alizé, TE: Trou d'Eau. Low density sites: CAP: Cap-Méchant, PTE: Petit Trou d'Eau. $S1_{cold}$: austral cold season 2019, $S2_{warm}$: austral warm season 2020, $S3_{cold}$: austral cold season 2020.

3.1.2. Clonal Diversity of *Stichopus chloronotus*

On the 216 individuals of *S. chloronotus* genotyped, 166 presented no missing data. From them, 19 MLGs were identified, of which 9 were shared between 2 and 48 individuals. The numbering of the MLGs cited in this study is the one used in the previous study [31]. Five MLGs seemed to be dominant: MLG34 was shared by 48 individuals, MLG05 was shared by 45, MLG02 by 22, MLG16 by 13 and finally MLG01 was shared by 15 individuals.

3.2. Clonal Propagation of Stichopus chloronotus through Space and Time

Sampling design highlighted a spatial heterogeneity among sites in the distribution of *S. chloronotus* clones; each site was characterized by its own dominant clones (Figure 2), with no MLG shared between both reefs. However, clonal distribution was stable over the three seasons ($S1_{cold}$, $S2_{warm}$ and $S3_{cold}$) as, for a given site, the same MLGs were found for each season (Figure 2). The clonal richness was higher in $S1_{cold}$ for all the sites: 0.75 for PAS, 0.29 for TE and 0.33 for ES (Table 4), but it may be explained by the low number of individuals that correctly amplified during genotyping. It was also higher for each and over the three seasons at PAS (Table 4), which is dominated by two MLGs: MLG01 representing 28% of the individuals sampled at this site, and MLG02 representing 40% of the individuals (Figure 2). Over all seasons, only one dominant MLG was found at TE (MLG34 representing overall 46% of the individuals) and at ES (MLG05 representing overall 71% of the individuals; Figure 2). For ES, in $S2_{warm}$, only two MLGs were found: MLG05 and MLG16, representing 83% and 17% of the individuals, respectively (Figure 2). The clonal richness was in consequence the lowest here (0.04; Table 4). PAS and TE, both located in the same reef complex, less than three kilometres apart, shared four MLGs: MLG01, MLG12, MLG34 and MLG37. No MLGs were shared between ES and the other sites, ES being located in another reef more than 20 kilometres southward (Figure 1). In conclusion, despite the spatial variability observed, there was no seasonal effect on the clonal distribution of *S. chloronotus*, nor any interannual effect during our monitoring; therefore, clonal propagation remained stable, as already found in [31].

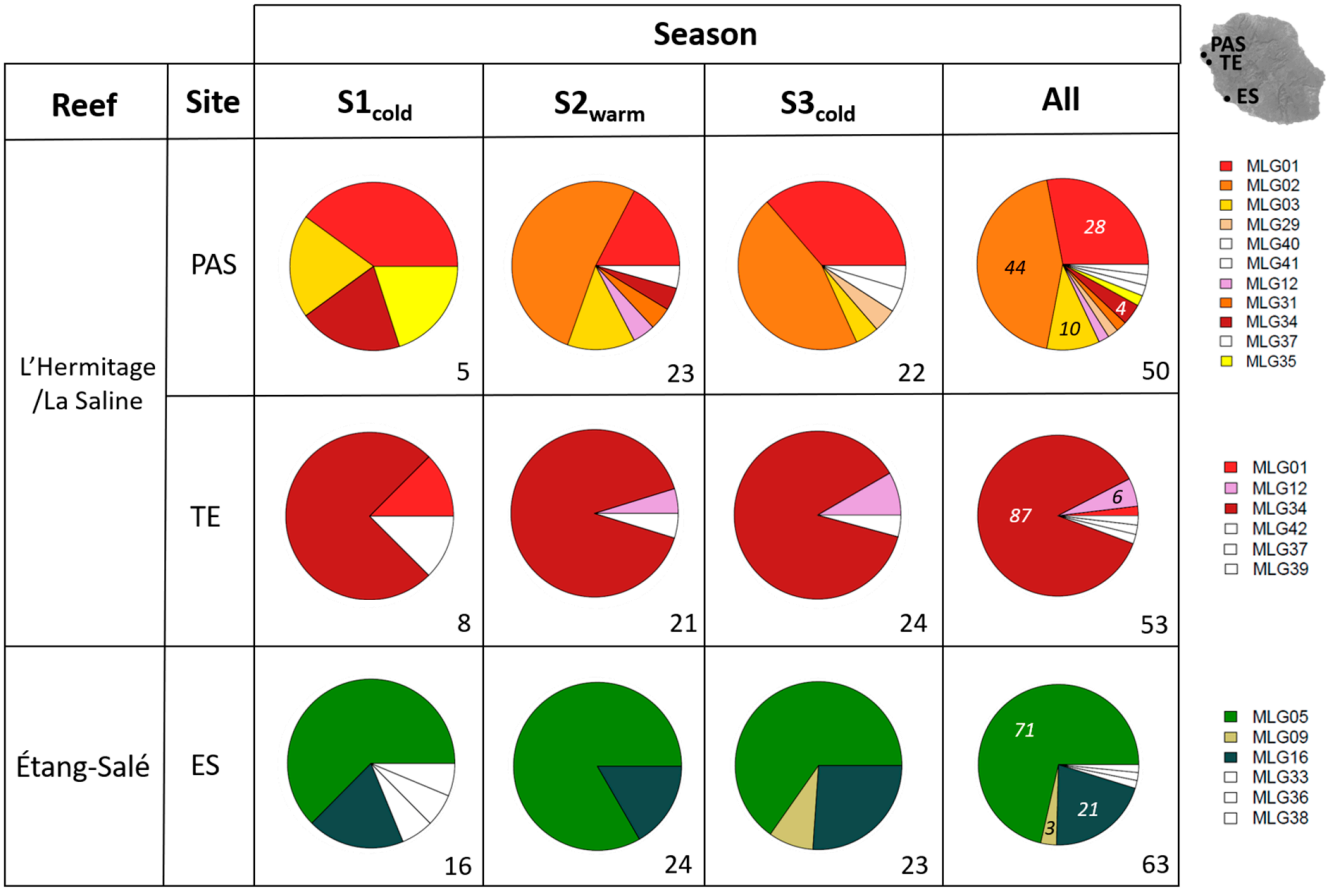

Figure 2. Spatial and temporal clonal distribution of *Stichopus chloronotus.* Numbers of individuals are indicated at the bottom right. MLGs are coloured following a previous study [31]. MLGs in white are unique over the previous study [31] and this study. Percentages of each MLG are indicated for the column "All". PAS: Passe de l'Ermitage, TE: Trou d'Eau, ES: Étang-Salé. $S1_{cold}$: austral cold season 2019, $S2_{warm}$: austral warm season 2020, $S3_{cold}$: austral cold season 2020.

Table 4. Indices of genetic diversity and clonal structure for *Stichopus chloronotus* populations from Reunion Island.

Season	Site	%*NA*	*N*	N_{MLG}	*R*	*Na*	*Np*	*Ho*	*He*	F_{IS}
S1	PAS	79.17	5	4	0.75	1.78 ± 0.22	0.11 ± 0.11	0.40 ± 0.13	0.28 ± 0.08	−0.44 *** ± 0.12
	TE	66.67	8	3	0.29	1.78 ± 0.32	0.11 ± 0.11	0.17 ± 0.10	0.16 ± 0.06	−0.02 ^{NS} ± 0.21
	ES	33.33	16	6	0.33	1.67 ± 0.17	0.22 ± 0.15	0.38 ± 0.13	0.26 ± 0.07	−0.50 *** ± 0.19
S2	PAS	4.17	23	7	0.27	1.78 ± 0.22	0.11 ± 0.11	0.25 ± 0.09	0.23 ± 0.07	−0.08 ^{NS} ± 0.12
	TE	12.50	21	3	0.10	1.78 ± 0.22	0.11 ± 0.11	0.12 ± 0.10	0.10 ± 0.05	−0.24 ** ± 0.24
	ES	0.00	24	2	0.04	1.56 ± 0.18	0.11 ± 0.11	0.41 ± 0.15	0.24 ± 0.08	−0.73 *** ± 0.11
S3	PAS	8.33	22	6	0.24	2.00 ± 0.33	0.22 ± 0.15	0.26 ± 0.09	0.20 ± 0.06	−0.29 *** ± 0.05
	TE	0.00	24	3	0.09	1.78 ± 0.22	0.00 ± 0.00	0.13 ± 0.10	0.12 ± 0.05	−0.02 ^{NS} ± 0.24
	ES	4.17	23	3	0.09	1.78 ± 0.15	0.11 ± 0.11	0.39 ± 0.12	0.26 ± 0.07	−0.50 *** ± 0.10

%*NA*: percentage of missing data from the initially sampling design due to genotyping difficulty; *N*: number of individuals with no missing data; N_{MLG}: number of distinct multi-locus genotypes; *R*: clonal richness; *Na*: mean number of alleles; *Np*: mean number of private alleles; *Ho* and *He*: observed and expected heterozygosities respectively; F_{IS}: inbreeding coefficient and significant deviations from Hardy–Weinberg Equilibrium (**: $p < 0.01$; ***: $p < 0.001$; ^{NS}: non-significant). Standard errors are indicated following means values. PAS: Passe de l'Ermitage, TE: Trou d'Eau, ES: Étang-Salé. $S1_{cold}$: austral cold season 2019, $S2_{warm}$: austral warm season 2020, $S3_{cold}$: austral cold season 2020.

3.3. Population Structure and Differentiation

The number of alleles per locus (*Na*) and the number of private alleles (*Np*) ranged between 0.29 ± 0.11 and 10.54 ± 0.72 for *H. leucospilota* (Table 3). On the contrary, *Na* and *Np* were very low and similar among all populations (site × season) for *S. chloronotus*, ranging between 1.56 ± 0.18 and 2.00 ± 0.33 and between 0.00 ± 0.00 and 0.22 ± 0.15, respectively (Table 4). The observed heterozygosity (*Ho*) and the expected heterozygosity (*He*) of *H. leucospilota* populations ranged between 0.39 ± 0.05 and 0.59 ± 0.04, and 0.79 ± 0.03 and 0.83 ± 0.02, respectively, and all sites deviated significantly from HWE (Table 3). For *S. chloronotus*, *Ho* and *He* ranged between 0.12 ± 0.10 and 0.41 ± 0.15, and 0.10 ± 0.05 and 0.28 ± 0.08, respectively, and almost all populations deviated significantly from HWE (Table 4).

Results from the Structure and DAPC assignments at $K = 2$ were not congruent for *H. leucospilota* (Figure 3), indicating that there is no genetic structure among the five sites nor among seasons. Results of the best *K* and BIC (Figure S2) were also not congruent. These results were well supported by the pairwise F_{ST} calculated between pairs of populations (Table 5) where only few were significantly different from zero.

Table 5. Genetic differentiation of *Holothuria leucospilota* populations with all individuals kept estimated with Weir and Cockerham's F_{ST}.

Season		$S1_{cold}$					$S2_{warm}$					$S3_{cold}$				
	Site	MNS	CAP	PLA	PTE	TE	MNS	CAP	PLA	PTE	TE	MNS	CAP	PLA	PTE	TE
$S1_{cold}$	MNS (10)	-														
	CAP (6)	0.007	-													
	PLA (15)	0.015	0.007	-												
	PTE (7)	0.033	0.028	0.017	-											
	TE (16)	0.020	0.040	0.035	0.039	-										
$S2_{warm}$	MNS (23)	0.018	0.015	0.010	0.005	**0.029** *	-									
	CAP (12)	0.045	0.036	**0.048** *	0.027	**0.066** ***	**0.029** *	-								
	PLA (24)	0.020	0.017	0.015	0.002	0.024	0.014	0.019	-							
	PTE (12)	0.033	0.021	0.026	0.006	**0.045** *	0.014	**0.044** ***	0.012	-						
	TE (23)	**0.042** *	0.028	**0.032** *	0.012	**0.040** *	0.012	**0.038** ***	0.013	0.009	-					
$S3_{cold}$	MNS (24)	0.029	0.031	**0.028** *	0.009	**0.042** ***	0.014	**0.032** *	0.016	0.007	0.013	-				
	CAP (12)	0.027	0.025	0.023	0.008	0.042	0.006	0.016	0.010	0.021	0.013	0.007	-			
	PLA (21)	0.026	0.015	0.012	0.009	**0.045** ***	0.009	0.015	0.012	0.021	0.017	0.013	0.005	-		
	PTE (12)	0.035	0.031	0.023	0.002	**0.045** *	0.015	0.023	0.009	0.019	0.011	0.004	0.003	0.011	-	
	TE (24)	0.018	0.032	0.017	0.009	**0.029** *	0.008	**0.044** ***	0.014	0.009	0.008	0.002	0.004	**0.019** *	0.008	-

p-values (*: $p < 0.5$; ***: $p < 0.001$) are indicated in bold. High density sites in red: MNS: Maître-Nageur-Sauveteur, PLA: Planch'Alizé, TE: Trou d'Eau. Low density sites in green: CAP: Cap-Méchant, PTE: Petit Trou d'Eau. For each population, *N* is indicated in parentheses.

Even if some clones were detected, i.e., individuals from the same MLG or MLL assigned to the same cluster, results from Structure and DAPC assignments at $K = 2$ were not congruent for *S. chloronotus* (Figure 4), as well as the results of the best *K* and BIC (Figure S3). However, keeping only one representative per MLG per site and pooling all seasons, DAPC showed a genetic differentiation between the reef of L'Hermitage/La Saline (PAS and TE) and the reef of Étang-Salé (ES) (Figure S4) for *S. chloronotus* populations. Results of pairwise F_{ST} for *S. chloronotus* revealed that ES was significantly genetically different from PAS and TE for each season (Table 6). It is congruent with the absence of shared MLGs observed between these sites. Few significant differences were observed among seasons in PAS and TE (Table 6). Moreover, no significant genetic differentiation was observed among seasons for each site (Table 6).

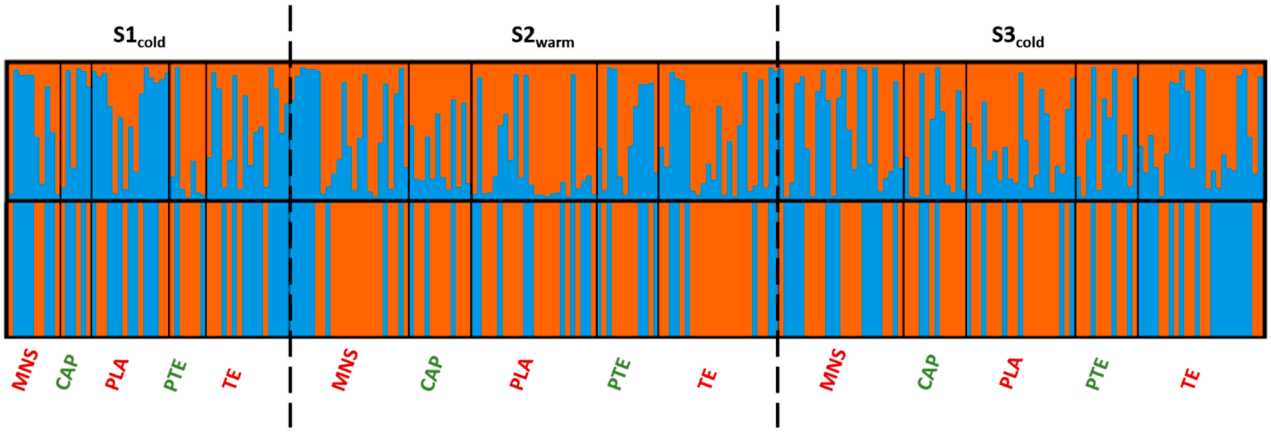

Figure 3. Assignment tests for *Holothuria leucospilota* individuals for the three seasons ($S1_{cold}$, $S2_{warm}$, $S3_{cold}$) and each site. Above: Structure plot at $K = 2$, and below: DAPC assignments at $K = 2$. High density sites in red: MNS: Maître-Nageur-Sauveteur, PLA: Planch'Alizé, TE: Trou d'Eau. Low density sites in green: CAP: Cap-Méchant, PTE: Petit Trou d'Eau.

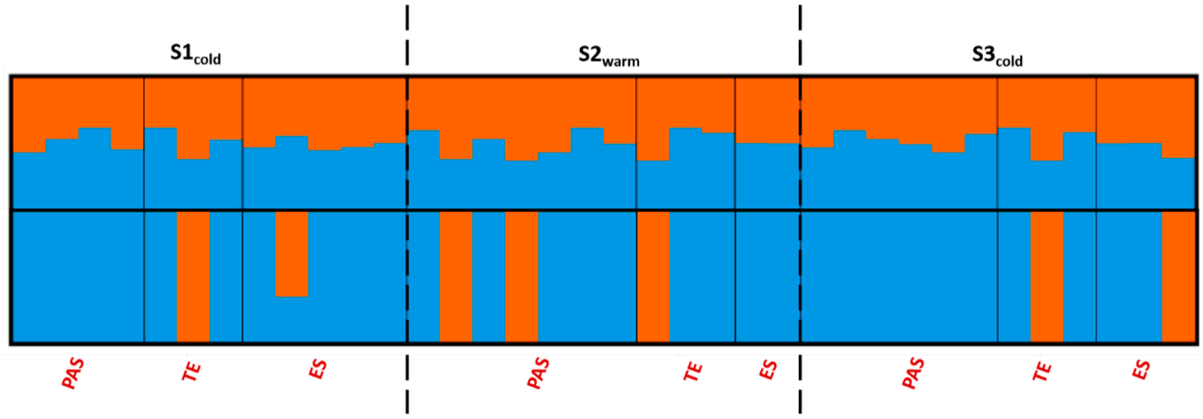

Figure 4. Assignment tests for *Stichopus chloronotus* individuals for the three seasons ($S1_{cold}$, $S2_{warm}$, $S3_{cold}$) and each site. Above: Structure plot at $K = 2$, and below: DAPC assignments at $K = 2$. PAS: Passe de l'Ermitage, TE: Trou d'Eau, ES: Étang-Salé.

Table 6. Genetic differentiation of *Stichopus chloronotus* populations with all individuals kept estimated with Weir and Cockerham's F_{ST}.

Season		**$S1_{cold}$**			**$S2_{warm}$**			**$S3_{cold}$**		
	Site	**PAS**	**TE**	**ES**	**PAS**	**TE**	**ES**	**PAS**	**TE**	**ES**
$S1_{cold}$	PAS (5)	-								
	TE (8)	0.002	-							
	ES (16)	**0.081** **	**0.113** ***	-						
$S2_{warm}$	PAS (23)	−0.002	0.064	**0.195** ***	-					
	TE (21)	**0.147** *	−0.016	**0.220** ***	**0.138** ***	-				
	ES (24)	**0.105** **	**0.142** ***	−0.016	**0.207** ***	**0.237** ***	-			
$S3_{cold}$	PAS (22)	0.017	**0.068** *	**0.232** ***	−0.004	**0.129** ***	**0.240** ***	-		
	TE (24)	0.095	−0.032	**0.188** ***	**0.117** **	−0.018	**0.209** ***	**0.111** ***	-	
	ES (23)	**0.071** *	**0.115** ***	−0.020	**0.189** ***	**0.219** ***	−0.007	**0.231** ***	**0.188** ***	-

p-values (*: $p < 0.5$; **: $p < 0.01$; ***: $p < 0.001$) are indicated in bold. PAS: Passe de l'Ermitage, TE: Trou d'Eau, ES: Étang-Salé. For each population, *N* is indicated in parentheses.

4. Discussion

4.1. Importance of the Sexual Reproduction for Holothuria leucospilota

Although both species exhibit the ability to reproduce sexually and asexually [27], their main mode of reproduction seems different. Results on MLGs and MLLs clearly indicate that there is no clone among the populations of *H. leucospilota* at any site nor season. It looks surprising regarding a previous study in Reunion Island where individuals undergoing fission were observed [23]; a fission rate of 5.2% was estimated by a visual census in Trou d'Eau (TE, herein). This fission rate is low compared to those estimated for *H. atra* on the same reef, ranging between 14.9% and 19.6% [46,47]. However, despite the high number of individuals undergoing fission estimated for *H. atra*, no clone was identified using microsatellite markers [32].

Several hypotheses may explain the absence of clones for *H. leucospilota*. First, only one study estimated the fission rate in *H. leucospilota* from Reunion Island [23], dated up to 25 years, and no genetic study concerning the reproduction of this species has been realised since then. Moreover, the lifespan of sea cucumbers in their natural habitat is a very problematic question, as no long-term capture-recapture method has yet been developed because of the rejection by tegument of any external tag [48]. For 25 years, sea cucumbers that have reproduced asexually may have died and sexual reproduction may have become the main mode of reproduction, leading to a high genetic diversity. Sexual reproduction of *H. leucospilota* seems to occur twice a year. The pattern of sexual reproduction in populations of *H. leucospilota* from Reunion Island has been investigated [49] using the gonad index and field observations. They observed that the first spawning event occurred in February and the second in May. Further studies found the same pattern for sexual reproduction of *H. leucospilota* in different localities, including Hong-Kong [50], Cook Islands [17] and Heron Island (Great Barrier Reef) [51]. However, the spawning of *H. leucospilota* occurred in a short period of two weeks in April in Darwin (Australia) [52]. Therefore, the relative rate of sexual reproduction compared to asexual reproduction seems to be much higher given the low rate of fission previously estimated [23] and the absence of clones in the population observed in this study. Sexual reproduction, favouring genetic mixing, could explain that individuals do not share MLG. Even the 1000 simulations, reducing genotyping to 9 microsatellite markers over 24, showed that no MLGs were shared among individuals. As a comparison, a previous study did not find any shared MLGs in the *H. atra* population [32], for which the number of alleles was high and in the same order of magnitude as for *H. leucospilota* (48 for *H. leucospilota* and 42 for *H. atra*). As a consequence, our results showed that *H. leucospilota* populations of Reunion Island have not use asexual reproduction for a long period.

Results of Structure and DAPC and the low values of F_{ST} between population pairs suggest that populations of *H. leucospilota*, whatever the site density (high or low), are weakly or not genetically differentiated. Therefore, populations of *H. leucospilota* throughout the fringing reef of L'Hermitage/La Saline are actually a metapopulation with larval/gametes exchanges within the reef. Moreover, this low genetic differentiation among sites is also found among seasons, meaning that sexual reproduction in *H. leucospilota* is stable through time in this part of the world.

4.2. Importance of Asexual Reproduction for Stichopus chloronotus

In contrast to *H. leucospilota*, individuals of *S. chloronotus* were grouped into few clones (only very few individuals presented a unique MLG). Asexual reproduction for *S. chloronotus* was already reported [24], using a visual census for detecting whether some individuals underwent fission. They revealed that the fission rate reached 16% in Trou d'Eau (TE herein), and fell to 0% in Étang-Salé (ES herein). Once again, we showed that genetic tools, such as microsatellite markers, seem more consistent to study clonal propagation than fission rate estimated by a visual census. In fact, only 6 MLGs were identified at Trou d'Eau (TE herein) and 6 others at Étang-Salé (ES herein), which were shared between 53 and 63 individuals, respectively.

Other studies used genetic tools allowing a comparison of the percentage of individuals sharing MLGs. In our study, we found that 94% of the individuals sampled shared MLGs all sites and seasons combined, as in [31], which reported 97%. Analyses using allozymes revealed that 95% of the individuals on the Great Barrier Reef (Australia) shared MLGs (i.e., $R = 0.24$) [53]. Using AFLP, 51 MLGs were identified within the 149 individuals sampled (i.e., $R = 0.34$), with up to 20 individuals presenting the same MLG [54]. Overall, the percentage of individuals sharing MLG is very high for *S. chloronotus* populations from different localities, meaning that asexual reproduction seems to occur at a very high rate, higher than sexual reproduction, which would lead to a higher genetic diversity.

Our results showed that PAS and TE, in the same reef, shared some MLGs but none with ES, located in another reef, meaning that clonal propagation is limited to the reef-scale. The same pattern was already observed in Reunion Island for sea cucumbers [31] and for corals [55]. However, differences in the dominant MLGs per site were observed through time, as in the previous study [31], except for PAS, where the same MLG (MLG02) was dominant. At ES, MLG05 is the MLG dominant in both studies, but MLG04, the previous second dominant MLG [31], was not identified in our study, replaced by MLG16, already found previously, but in few individuals [31]. The number of individuals sampled in both studies was different, with on average 64 and 24 individuals for the previous study [31] and our study, respectively, due to a change of the density from 2.3 ± 0.2 ind·m^{-2} [31] to 0.7 ± 0.1 ind·m^{-2} (unpublished data). This decrease in density may have led to the loss of clones presenting a weaker fitness, explaining the variation in dominant MLGs observed between both studies. For TE, there is a clear shift of the dominant MLG between both studies; MLG01 was dominant between 2013 and 2016 [31], but it was only identified in one individual in our study, where MLG34 dominated.

Clonal propagation is stable through seasons. A previous study on clonal propagation of *S. chloronotus* from Reunion Island also showed no difference in the composition of MLGs within populations over four seasons [31]. Asexual reproduction of *S. chloronotus* in Reunion Island reaches a maximum level in the end of austral winter in October, with the highest fission rate of about 24% [24]. More recently, in winter 2013, the fission rate of *S. chloronotus* in Reunion Island has quietly decreased and reached 11.5% (P. Frouin, unpublished data). The fission rate reached 31% in July for the population at the Great Barrier Reef (Australia) [56]. Therefore, asexual reproduction occurs often in the cold season, where the environmental conditions are the less favourable, and sexual reproduction in the warm season [24,56]. As we found a temporal stability in the number of MLGs through seasons, sexual reproduction might occur but at very low rate, and asexual reproduction is the main mode of reproduction for *S. chloronotus* in Reunion Island.

4.3. Differences in Reproductive Strategies in Two Sympatric Sea Cucumber Species

Sympatric species share the same biotic and abiotic conditions. Here, two sea cucumbers species, *H. leucospilota* and *S. chloronotus*, have a patchy distribution with high density in the reefs of Reunion Island. We showed that these two species, while both able to reproduce sexually and asexually through fission, tend to use distinct reproductive modes and above all, at different seasons: sexual reproduction through gamete spawning in the warm season for *H. leucospilota* and clonal reproduction by transversal fission in the cold season for *S. chloronotus*.

Some studies on sea cucumbers have already shown that sympatric species that theoretically are able to reproduce asexually do not always do so [51,57]. For instance, *Holothuria atra* individuals underwent fission whereas *H. leucospilota* did not in Marshall Islands [57]. Additionally, this difference in reproductive strategies has already been highlighted for other marine sympatric species, such as sea stars. For example, *Leptasterias hexactis* and *Pisaster ochraceus*, both sympatric in San Juan Island (USA), have two distinct strategies of reproduction: the first broods few and large youths in the brood chamber in winter, whereas the second broadcasts many small eggs in spring, reducing the interspecific competition for habitat and food resources [58]. Moreover, the coral genus *Pocillopora*

includes broadcast spawners and brooders, which can be frequently found in the same reef [59].

Another reproductive strategy for reducing interspecific competition with the same reproductive mode is to alternate the period of reproduction. Analyses of gonadosomatic index revealed that two sympatric species of crabs in Guanabara Bay (Brazil) have a seasonal and alternative reproductive peak, with *Callinectes danae* reproducing in autumn and winter and *Callinectes ornatus* in spring and summer [60]. Authors concluded that the reproductive strategies of the two species of crabs leads to the avoidance of direct interspecific competition for available resources for planktonic larvae. Moreover, two sympatric species of sponges brood at two different times in the year, with *Dysidea avara* in June and July and *Phorbas tenacior* from August to October, avoiding overlap of the larval release period [61].

Therefore, our study highlights that these two sympatric sea cucumber species from Reunion Island use different reproductive strategies at different periods of the year: asexual reproduction in the cold season for *S. chloronotus* and sexual reproduction in the warm season for *H. leucospilota*. This non-overlapping of reproductive periods helps to reduce the interspecific competition for both food resources and habitat space. In fact, sexual reproduction leads to planktotrophic larvae, which migrate with the current, whereas asexual reproduction produces twice as many small individuals, but still in the adult stage, which are deposit-feeders, remaining in the same high-density patch into the reef. Additionally, even if post-settled sea cucumbers from sexual reproduction can be found near adult patches, they do not exhibit the same behaviour as adults and do not feed on the same food resources until they reach a specific size [62].

5. Conclusions

This study highlights that two sympatric sea cucumber species from Reunion Island that have the ability to reproduce both sexually and asexually (by fission), each using one of these strategies of reproduction preferentially. *Holothuria leucospilota* reproduces sexually whereas *S. chloronotus* reproduces mainly asexually. Therefore, there is no overlap in the reproduction periods of the two species, as both modes of reproduction occur in different seasons. These two different strategies of reproduction drastically reduce the interspecific competition for food and habitat, in a context of hyperdensity. Knowledge on the ecology and genetic structure and diversity of these two sea cucumber species will be very useful for aquaculture purposes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15050670/s1, Figure S1: Distribution of pairwise differences (number of alleles shared) among multi-locus genotypes (MLG) for *Holothuria leucospilota*; Figure S2: Results of the assignment tests for *Holothuria leucospilota*. (a) Mean likelihood over the five iterations of the same *K*, (b) Evanno's Δ*K* distribution, (c) BIC distribution and (d) plots from *K* = 2 to *K* = 5 for Structure (above) and DAPC (below); Figure S3: Results of the assignment tests for *Stichopus chloronotus*. (a) Mean likelihood over the five iterations of the same *K*, (b) Evanno's Δ*K* distribution, (c) BIC distribution and (d) plots from *K* = 2 to *K* = 5 for Structure (above) and DAPC (below); Figure S4: Results of the assignment tests for *Stichopus chloronotus* keeping only one representative per MLG, for each site, all seasons pooled. (a) Mean likelihood over the five iterations of the same *K*, (b) Evanno's Δ*K* distribution, (c) BIC distribution and (d) plots from *K* = 2 to *K* = 5 for Structure (above) and DAPC (below); Table S1: Summary of the sampling design.

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