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Genome-wide comparison of ultraviolet and ethyl methanesulphonate mutagenesis methods for the brown alga *Ectocarpus*

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Abstract

Ectocarpus has emerged as a model organism for the brown algae and a broad range of genetic and genomic resources are being generated for this species. The aim of the work presented here was to evaluate two mutagenesis protocols based on ultraviolet irradiation and ethyl methanesulphonate treatment using genome resequencing to measure the number, type and distribution of mutations generated by the two methods. Ultraviolet irradiation generated a greater number of genetic lesions than ethyl methanesulphonate treatment, with more than 400 mutations being detected in the genome of the mutagenised individual. This study therefore confirms that the ultraviolet mutagenesis protocol is suitable for approaches that require a high density of mutations, such as saturation mutagenesis or Targeting Induced Local Lesions in Genomes (TILLING).

Keywords: brown algae, *Ectocarpus*, ethyl methanesulphonate, mutagenesis, ultraviolet

Abbreviations: EMS, ethyl methanesulphonate; UV, ultraviolet, SNP, single nucleotide polymorphism, CDS, coding sequence

1. Introduction

The filamentous brown alga *Ectocarpus* has been the object of phycological research since the nineteenth century and has played a central role in several important discoveries, including for example the characterisation of brown algal pheromones, the discovery of brown algal viruses and the characterisation of its UV sexual system [1-6]. These advances, together with several features of this organism such as its small size and its capacity to complete its life cycle under laboratory conditions, led to *Ectocarpus* being proposed as a model organism for the brown algae in 2004 [7]. Since this initial proposal, a number of genomic resources have been developed for this organism, the most notable being the publication of a complete genome sequence in 2010 [8]. In addition, considerable effort has been put into creating genetic resources for *Ectocarpus* and the resources currently available include mutagenesis and classical genetic protocols, a genetic map and inbred lines [2, 9-11]. These genetic tools are currently being used to address a number of questions about brown algal biology, including for example life cycle regulation, sex determination and regulation of morphogenesis [2, 9, 12, 13]. Several of these studies involve the analysis of genetic mutants.

Ultraviolet (UV-C) irradiation of gametes is currently the most widely used method for generating genetic mutants in *Ectocarpus*. However, the current protocol has been optimised based on dose lethality and very little is known about the genomic effects of irradiation in terms of the number and types of mutations that are induced. Moreover, UV-C irradiation has not been compared with alternative, chemical mutagenesis approaches that have proved to be highly efficient in other model systems. For example, chemical mutagenesis with the alkylating agent ethyl methanesulphonate (EMS) has been shown to efficiently induce mutations in the flowering plant model *Arabidopsis* [14, 15].

The objective of the work reported here was to compare optimised UV-C and EMS mutagenesis protocols for *Ectocarpus* by directly analysing the genetic lesions caused by the two mutagens using genome-wide, sequence-based analysis of mutant individuals. The results of these analyses indicated that UV-C mutagenesis generates a higher density of genetic lesions per individual than EMS mutagenesis. Moreover, based on the number of mutations generated (more than 400 per individual) UV-C mutagenesis should be suitable for approaches that require collections of individuals each carrying a large number of mutations such as saturation mutagenesis or Targeting Induced Local Lesions in Genomes (TILLING) [16]. We also describe the construction of a strain adapted for these and other mutagenesis-based approaches.

2. Results and discussion

2.1. Construction of an optimised strain for mutagenesis and mutant analysis

Conditions have been defined for the completion of the *Ectocarpus* life cycle under laboratory conditions [17, 18] but difficulties can be encountered during culture depending on the strain being grown. This is particularly the case when working with mutant lines, which may exhibit marked morphological or physiological modifications. Problems are most often encountered at the stage when the sporophyte produces the unilocular sporangia in which meiosis occurs. To circumvent this problem, we constructed a mutant strain carrying recessive mutations that maintain it in the gametophyte generation. For this, both the *ouroboros* (*oro* [9]) and *immediate upright* (*imm* [12]) mutations were introduced into the same strain and, in the process, two backcrosses were carried out to reduce the number of additional mutations carried by the strain (see Fig. 1 and the Methods section for details). The *oro* and *imm* mutations cause this strain (designated Ec197-21) to continually reiterate the gametophyte generation so that any mutants generated can be directly crossed for genetic analysis (genetic mapping, complementation tests, etc.). This is an advantage over wild type strains, which are maintained as the sporophyte generation, making it necessary to induce the production of unilocular sporangia and transition to the gametophyte generation before the strains can be crossed (Fig. 2). As the *oro* and *imm* mutations are recessive, any diploid progeny produced by crossing can express the sporophyte program and therefore progress through the life cycle, allowing further genetic manipulations such as the generation of segregating populations for gene mapping (Fig. 2).

2.2. Optimisation of the EMS mutagenesis protocol

Strain Ec197-21 was mutagenised using UV-C irradiation and treatment with the chemical mutagen EMS in order to compare the efficacy of the two mutagenesis methods. UV-C mutagenesis was carried out using the protocol described by Coelho *et al*. [9] except that it was necessary to adjust the period of irradiation to 30 min to obtain a lethality of 50%.

To optimise the EMS mutagenesis protocol, Ec197-21 gametes were treated with different concentrations of EMS for either five or 16 hours at 13°C (Table 1). Based on gamete lethality, treatment with 0.25% v/v EMS for 16 hours was selected as the optimal treatment.

2.3. Sequence analysis of UV-C and EMS mutagenised individuals

To assess and compare the effects of UV-C and EMS mutagenesis at the genome level, large-scale mutagenesis experiments were carried out using either irradiation with UV-C light for 30 minutes or treatment with 0.25% v/v EMS for 16 hours. One individual was then selected at random from each of the two mutagenic treatments (named 20-1 and 18-1 for the UV-C and EMS treatments, respectively) and multiplied clonally for DNA extraction. Genome resequencing was carried out by generating 12,455,830,634 bp (58x coverage) and 10,668,189,461 bp (50x coverage) of Illumina HiSeq2500 125 bp paired-end sequence data for the UV-C and EMS mutated individuals, respectively. Cleaned and trimmed sequence data for the two samples were optimally mapped to the *Ectocarpus* sp. strain Ec32 reference genome using Bowtie2 and the GATK suite and sequence variants were called using three different algorithms: the Samtools programs mpileup and bcftools view [19], SHORE qVar [20] and GATK UnifiedGenotyper [21]. We only retained variants identified by at least two of these algorithms. Moreover, to limit the detection of false positive mutations, only variants with sequence coverage of between 20x and 50x were retained and filters were also applied for Phred-scaled variant quality score (50) and variant frequency (0.95). Polymorphisms present in the parent strains were identified and eliminated by mapping Illumina sequence data for the female parent Ec597 [2] onto the male parent (Ec32) reference genome [8]. Finally, all variants that were found in both the UV-C and the EMS mutagenised individuals were eliminated as we considered it extremely unlikely that identical mutations would be produced in the two strains and these more likely corresponded to errors in the Ec32 reference sequence.

Resequencing detected a total of 442 and 129 mutations in the UV-C and EMS mutagenised individuals, respectively (Table 2). UV-C irradiation and EMS treatment therefore induced one mutation every 375 kbp (a frequency of $2.67e^{-6}$) and 1,296 kbp (a frequency of $7.72e^{-7}$), respectively (Table 3). These counts probably underestimate the number of mutations in the genomes of the two individuals to some extent because only just over 84% of the genome was sequenced at a coverage of 20-50x in both cases and mutations in the remaining 16% of the genome would not have been detected or would have been discarded. Based on this analysis, however, the UV-C mutagenesis protocol clearly produced a higher number of mutations per individual than EMS mutagenesis.

Twenty-six of the UV-induced mutations were selected at random and further analysed by PCR amplification and Sanger sequencing of the mutated region. All but four of the 26 mutations were confirmed by this approach. Of the four mutations that were not confirmed, two corresponded either to sequencing errors in the reference sequence or to polymorphisms that were already present in the Ec197-21 strain before mutagenesis and two to loci where variation from the reference sequence was not confirmed by Sanger sequencing. The Sanger sequencing analysis therefore confirmed 84.6% of the mutations detect by the Illumina resequencing pipeline. Extrapolating this confirmation rate to the entire set of mutations detected by the latter approach, we predict that approximately 374 of the 442 mutations would be validated by Sanger sequencing (corresponding to a mutation rate of $2.26e^{-6}$ per base). However, note again that the Illumina approach was limited to 84% of the genome (regions with 20x to 50x coverage) and therefore would not have detected all the mutations induced by the UV-C treatment. Overall, therefore, the Sanger sequencing analysis indicated that the large majority of the variants detected by the Illumina resequencing pipeline were *bona fide* mutations.

EMS has been used extensively to generate large mutant collections for flowering plant species and, consequently, a considerable amount of information is available about the density of mutations induced by treatment with this mutagen. Frequencies of EMS-induced mutations of between $1e^{-4}$ to $1e^{-6}$ have been observed in these studies, corresponding to one mutation every 400 kbp in *Arabidopsis* [22], every 200 kbp in *Pisum sativum* [23] and every 146 kbp to 848 kbp in Melon [24]. The mutation frequency observed in the present study was lower than that reported in these previous studies. This difference may have been due to a number of factors. First, it is possible that, despite the precautions taken to minimise instability of the EMS during the mutagenic treatment (seawater buffered at pH 7, treatment at a low temperature of 13°C and incubation in the dark), the molecule may have lost some of its potency over time in the seawater medium. Second, in the absence of an easily scored recurring phenotype, lethality was used to optimise the mutagenic dose (50% lethality, i.e. similar to the lethality rates observed with flowering plants; Table 1), and it is possible that this indirect method of measuring the effect of the mutagen did not result in an optimal treatment regime.

EMS has been shown to predominantly induce point mutations (mainly guanine to adenine) through alkylation of guanine residues [15, 25, 26]. Only 14% of the mutations detected in strain 18-1 were of this type, suggesting that part of the EMS mutagenic action may have been through indirect, non-canonical effects due, for example, to toxicity.

UV radiation is known to exert at least part of its mutagenic effect via the formation of covalent bonds between adjacent pyrimidine nucleotides (formation of dipyrimidines), which result principally in cytosine to thymine transitions (C>T or CC>TT [27]). However, UV irradiation can induce other types of point mutation and the formation of dipyrimidines can lead to double-strand DNA breaks during DNA replication, resulting in deletions [28]. A significant proportion (62.4%) of the mutations detected in the genome of strain 20-1 corresponded to UV signature mutations; we detected one UV-signature mutation per 600 kbp (or a mutation frequency of $1.6e^{-6}$ per base).

Detailed analysis of the 442 UV-induced mutations showed that 58 (13.1%) were located in the coding regions of genes and that 36 (8.1%) of these mutations modified the coding region of the gene by causing either missense or nonsense mutations.

2.4. Conclusions

This study demonstrated, based on genome resequencing, that UV-C mutagenesis of *Ectocarpus* gametes results in a large number of genetic lesions, 442 in the randomly selected individual analysed here. The majority of these mutations are SNPs and the types of mutation observed are consistent with what is known about the mode of action of this mutagen. UV-C mutagenesis was found to generate genetic lesions more effectively than chemical mutagenesis with EMS. The number of mutations observed per individual is sufficient to envisage genome-wide mutagenesis approaches such as saturation mutagenesis and TILLING. For example, based on the 58 mutations detected in coding regions in the 20-1 strain, a population of 2000 UV-mutagenised individuals could be expected to contain approximately 116,000 CDS mutations or more than seven mutant alleles for each gene in the *Ectocarpus* genome.

3. Methods

3.1. *Ectocarpus* strains and culture

Both UV-C and EMS mutagenesis was carried out on a strain (Ec197-21) that carried both the *oro* [9] and the *imm* [12] mutations but which had been backcrossed twice to reduce the number of mutations elsewhere in the genome (Fig. 1). For this, the original *oro* mutant strain (Ec494), which is a UV-mutagenised, clonal descendant of the genome-sequenced strain Ec32 [8], was crossed with a female line carrying the *imm* mutation (Ec419). Ec419 had been derived by crossing the original, spontaneous *imm* mutant line, Ec137, with a sister, Ec25. Both Ec137 and Ec25 are siblings of the genome-sequenced strain Ec32. The diploid sporophyte (Ec566) derived from the cross between Ec494 and Ec419 gave rise to a female *oro imm* gametophyte (Ec597) that was backcrossed with Ec32 to further remove UV-induced mutations other than *oro*. This cross produced a diploid sporophyte, Ec197, which gave rise to the female *oro imm* backcrossed strain, Ec197-21, that was used for the mutagenesis experiments. Strains were cultivated under standard conditions [18]. Strain Ec197-21 is available on request.

3.2. UV and EMS mutagenesis of *Ectocarpus* gametes

UV mutagenesis was carried out as described by Coelho et al. [9] except that the gametes were irradiated for 30 min rather than 45 min. Briefly, gametes were irradiated with a UV (254 nm) lamp for 30 min immediately after release from plurilocular gametangia. Irradiated gametes were allowed to settle in the dark at 13 °C for 4 h. Petri dishes were then transferred to a culture chamber at 13 °C and cultivated as described above. For the

EMS mutagenesis, gametes were released synchronously, as described, into natural, unsupplemented seawater and about 100 µl of gametes were immediately diluted in 2 ml of Tris-buffered (100mM Tris-HCl pH 7) natural seawater containing different concentrations of EMS. After incubation for either five or 16 hours at 13°C in the dark, the gametes were washed three times with 80 ml of Tris-buffered natural seawater for about 30 min with gentle shaking.

3.3. Genome resequencing and identification of mutations

Genome resequencing was carried out by generating 125 bp paired-end reads using Illumina HiSeq2500 technology (Fasteris, Switzerland). Raw sequence data were cleaned and trimmed using Prinseq [29]. Reads were trimmed from both ends to remove nucleotides with quality less than 20 and reads were then only retained if they were longer than 50 nucleotides, had a mean quality of at least 25 and no non-determined nucleotides. Reads were mapped to the Ec32 reference genome using Bowtie2 [30] and the Indels Realigner and Base Score Recalibration programs of the GATK suite [21, 31] were used to improve read alignment and quality parameters, respectively. Sequencing depth per base was estimated using the Samtools depth program and the estimation used to determine a relevant sequencing coverage working interval. Variants were then identified by independently running three different variant-calling programs: Samtools mpileup and bcftools, SHORE qVar and the GATK UnifiedGenotyper. Variants were called for each of the mutagenised strains (the EMS mutagenised strain 18-1 and the UV mutagenised strain 20-1) and for the female parental strain Ec597. A number of filters were applied in order to retain only high quality variants. These involved selecting only variant loci where 1) coverage was to a depth of between 20 and 50, 2) the variant sequence was at a frequency of 0.95 or higher and 3) the Phred-scaled variant quality score was over 50. These filters were either applied during variant calling (SHORE qVar) or afterwards (Samtools mpileup and Unified Genotyper) using bcftools. The VFCtools suite was then used (vcf-isec command) to compare vcf files and remove variants shared by two or more strains in order to retain only variants that were unique to each mutant strain. A list of putative mutations was established for each of the two mutants by comparing the results from the three variant calling programs and retaining only variants that had been identified by at least two programs. Twenty-six variants were then randomly selected from this list of putative mutations and further analysed by PCR amplification of the local genomic region (1.5 mM $MgCl_2$, 0.25 mM each dNTP, 0.5 mM each oligonucleotide primer [Eurogentec], 5% DMSO, 0.05 U/µl GoTaq® DNApolymerase [Promega, USA]) and Sanger sequencing of the amplified product (MWG Eurofins, Germany). PCR primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and Sanger sequences were analysed using the CodonCodeAligner software (http://www.codoncode.com/aligner/).

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Tables

Table 1

Optimisation of the EMS mutagenesis protocol.

EMS concentration (% v/v)	Duration of treatment (h)	Estimated lethality (% kill)
0	5	0
0.5	5	25
1.0	5	70
0	16	0
0.25	16	43
0.5	16	21

Table 2

Detection of UV-C- and EMS-induced mutations by genome resequencing. Underlined numbers indicate mutagen signature mutations. SNP, single nucleotide polymorphism; CDS, coding sequence.

Mutagen		**EMS**			**UV-C**		
Total identified mutations		**129**			**442**		
Insertions (average size nt)		5 (2.2)		3.88%	8 (2.6)		1.81%
Deletions (average size nt)		8 (3.5)		6.20%	7 (11)		1.58%
SNPs		116		89.92%	427		96.61%
transversion	C>T	10	18	13.95%	149	276	62.44%
	G>A	8			127		
	A>G	15	30	23.26%	24	64	14.48%
	T>C	15			40		
	A>C	4	9	6.98%	17	28	6.33%
	T>G	5			11		
	C>A	9	22	17.05%	13	26	5.88%
	G>T	13			13		
transition	A>T	6	10	7.75%	9	19	4.30%
	T>A	4			10		
	C>G	18	27	20.93%	7	14	3.17%
	G>C	9			7		
CDS mutations		**7**		**5.43%**	**58**		**13.12%**
silent		2		1.55%	22		4.98%
missense		5		3.88%	35		7.92%
nonsense		0		0.00%	1		0.23%

Table 3

Number and density of mutations by class

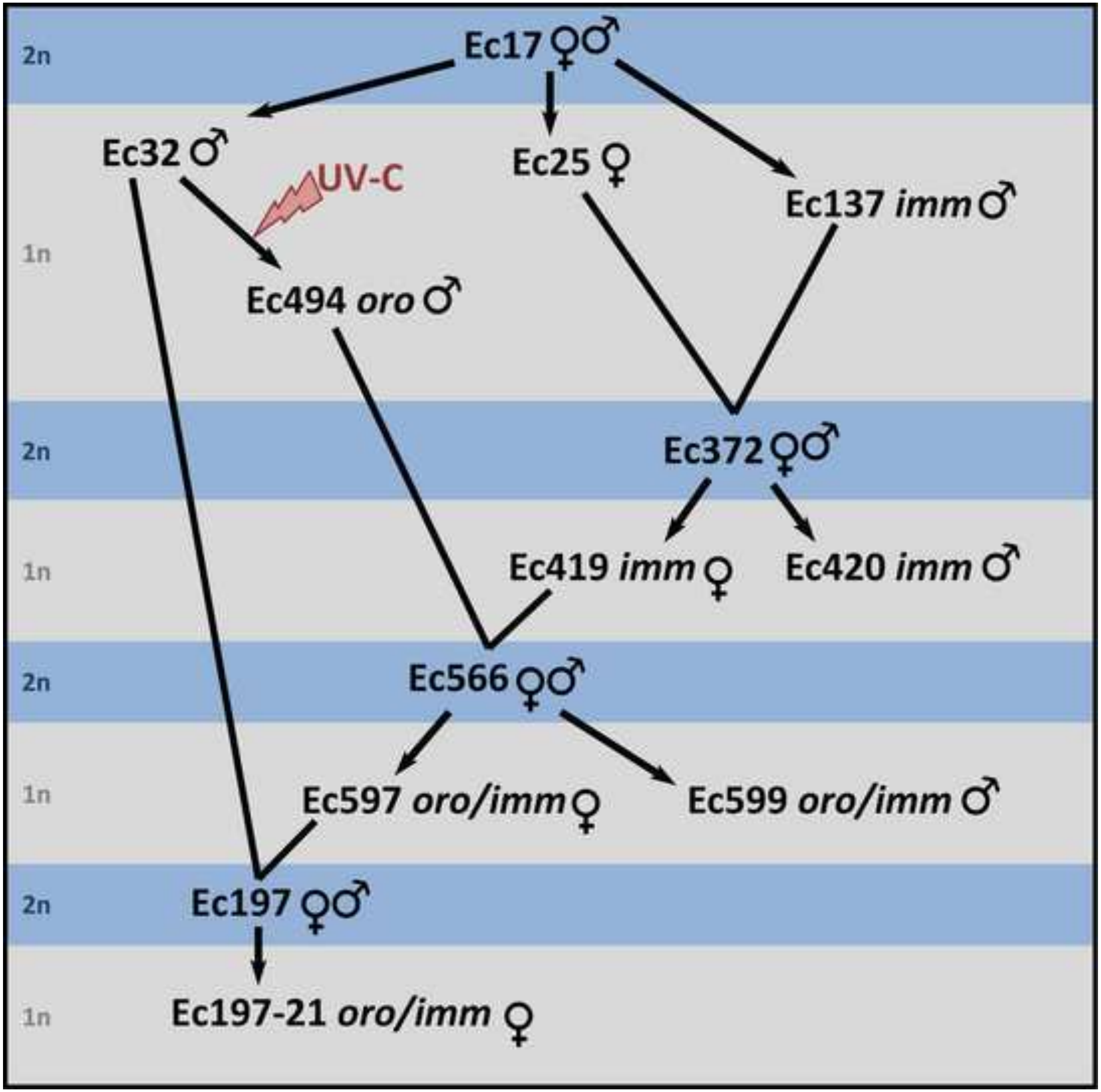
Mutant strain	Mutation class	Number of mutations	Average interval between mutations (kbp)	Mutation frequency (per base)
18.1 mutant (EMS)	**Total**	**129**	**1296**	**$7.72e^{-7}$**
	SNPs	116	1441	$6.94e^{-7}$
	Mutagen signature mutations	18	9287	$1.08e^{-7}$
	Insertions	5	33434	$2.99e^{-8}$
	Deletions	8	20896	$4.79e^{-8}$
20.1 mutant (UV-C)	**Total**	**442**	**375**	**$2.67e^{-6}$**
	SNPs	427	388	$2.58e^{-6}$
	Mutagen signature mutations	276	600	$1.66e^{-6}$
	Insertions	7	23685	$4.22e^{-8}$
	Deletions	8	20724	$4.83e^{-8}$

Figures

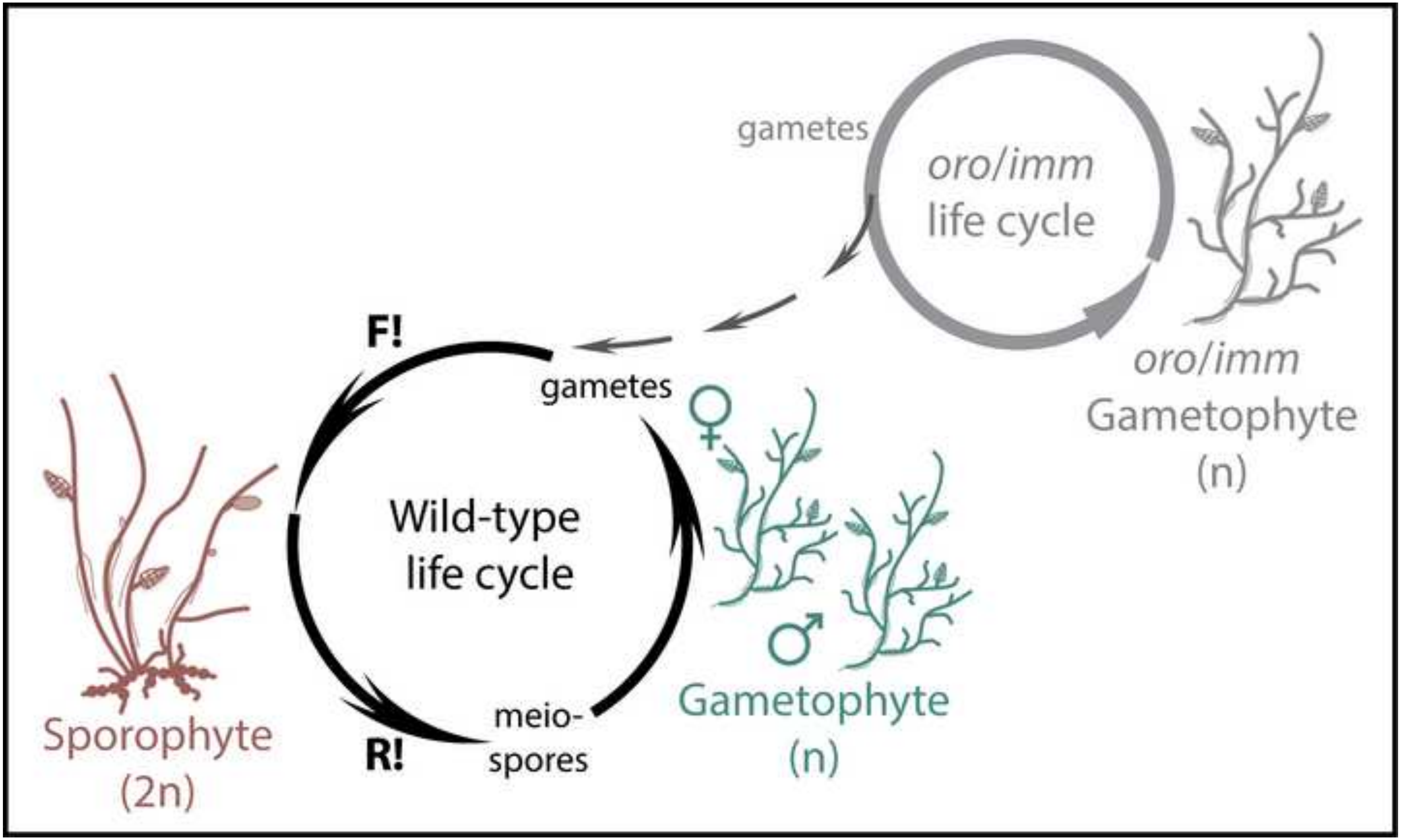
Fig. 1. Construction of strain Ec197-21, which is optimised for the TILLING protocol. See section 3.2 for details. UV-C, ultraviolet mutagenesis; 1n, haploid, 2n, diploid.

Fig. 2. Life cycles of wild type and *oro imm* double mutant *Ectocarpus* strains. The wild type *Ectocarpus* life cycle (in black) involves alternation between a diploid sporophyte generation and a haploid, dioicous gametophyte generation. Haploid meio-spores are produced via meiosis (R!) in unilocular sporangia on the sporophyte and these spore develop into gametophytes following release. Gametophytes produce either male or female gametes, which fuse with a gamete of the opposite sex to produce a zygote (F!), the diploid initial cell of the next sporophyte generation. The *oro imm* double mutant cannot initiate the sporophyte program and its gametes therefore reiteratively develop parthenogenetically as gametophytes (in grey). However, as the *oro* and *imm* mutations are recessive, zygotes formed by fusion with wild type gametes are able to initiate the sporophyte program and enter the normal life cycle (dark grey arrows). n, haploid, 2n, diploid.

Figure


2n
Ec17 ♀♂
1n
Ec32 ♂
UV-C
Ec25 ♀
Ec137 *imm* ♂
Ec494 *oro* ♂
2n
Ec372 ♀♂
1n
Ec419 *imm* ♀
Ec420 *imm* ♂
2n
Ec566 ♀♂
1n
Ec597 *oro/imm* ♀
Ec599 *oro/imm* ♂
2n
Ec197 ♀♂
1n
Ec197-21 *oro/imm* ♀

Figure


gametes
oro/imm
life cycle
oro/imm
Gametophyte
(n)
F!
gametes
Wild-type
life cycle
♀
♂
Sporophyte
(2n)
R!
meio-
spores
Gametophyte
(n)