Linkage Mapping Identifies the Sex Determining Region as a Single Locus in the Pennate Diatom *Seminavis robusta*

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**Abstract**

The pennate diatom *Seminavis robusta*, characterized by an archetypical diatom life cycle including a heterothallic mating system, is emerging as a model system for studying the molecular regulation of the diatom cell and life cycle. One of its main advantages compared with other diatom model systems is that sexual crosses can be made routinely, offering unprecedented possibilities for forward genetics. To date, nothing is known about the genetic basis of sex determination in diatoms. Here, we report on the construction of mating type-specific linkage maps for *S. robusta*, and use them to identify a single locus sex determination system in this diatom. We identified 13 mating type plus and 15 mating type minus linkage groups obtained from the analysis of 463 AFLP markers segregating in a full-sib family, covering 963.7 and 972.2 cM, respectively. Five linkage group pairs could be identified as putative homologues. The mating type phenotype mapped as a monogenic trait, disclosing the mating type plus as the heterogametic sex. This study provides the first evidence for a genetic sex determining mechanism in a diatom.

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**Introduction**

Diatoms (Bacillariophyceae) belong to the Stramenopila, which comprise several microalgal groups dominating primary production in aquatic environments [1]. The diatoms are one of the most diverse and productive groups of algae, with an estimated 200,000 species responsible for almost 20% of global primary production [2]. They are also promising from a biotechnological point of view, and hold great potential for the production of high-value bioproducts such as lipids, pigments and biofuels [3]. The available genomic resources for diatoms have grown rapidly over the past few years [4,5]. In addition, tools for reverse genetics have been developed [6]. However, sexual reproduction, a key feature of most diatom life cycles [7], has never been demonstrated for the most commonly used model diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* [3]. This prevents the use of forward genetics to link phenotype to genotype, including the use of mutagenesis and QTL mapping [8].

Life cycles, including sexual reproduction, have been studied in detail for only a minute fraction of known diatom species, but these represent most principal diatom lineages. The diatom life cycle comprises two main stages: a prolonged vegetative stage lasting months to years, which is diploid, and a short sexual stage lasting hours to days [3,7,9]. During the vegetative stage of the life cycle, a gradual reduction in cell size takes place, caused by physical constraints imposed by their silica cell wall. This cell wall comprises two parts (or thecae), one of which (the epitheca) is slightly larger than, and overlaps, the other (the hypotheca). During mitosis, each daughter cell inherits one maternal valve (which becomes the new epitheca) and de novo synthesizes a smaller hypotheca. Below a species-specific size threshold (the sexual size threshold, SST) cells become capable of sexual reproduction. Restoration of the maximal cell size generally occurs through sexual reproduction. The zygote matures into an auxospore, which expands to on average two to three times the size of the parental cells. After reaching its maximum size, a new so-called initial cell is formed inside the auxospore envelope, initiating a new round of vegetative multiplication. Because of its crucial role in cell size restoration, sexual reproduction is an obligatory stage in the life cycle of most diatoms.

The raphid pennate diatom *S. robusta* has recently been advocated as a model organism to study diatom biology, and in particular life cycle regulation [3]. Like most pennate diatoms [7,10–12], *S. robusta* has a heterothallic mating system with two mating types (MT+ and MT−) [13,14]. After cell pairing, each of the two cells forms two morphologically and behaviorally identical gametes. Subsequent zygote formation and auxospore expansion finally result in two initial cells with a cell length of 64–73 μm. Sexual reproduction is easily induced in cultured strains with a cell size below the SST by adding a suitable mating partner which allows sex to be reliably controlled in mating experiments. However, as in some other pennate diatoms (*e.g. T. tabulata* and *F. delicatissima*) [12,15], intraclonal reproducion (homothally) can
sporadically occur (in S. robusta in MT+). Additional advantages of S. robusta are that the cells are reasonably large (SST ~50 µm apical cell length) and can grow on surfaces allowing easy non-intrusive monitoring of cell and life cycle stages using inverted microscopy.

To date, genetic maps exist for only a small number of species outside the opisthokont and Plantae lineages. Within the stramenopile lineage, linkage maps have been reported for the brown alga *Ectocarpus siliculosus* [16] and several oomycete species [17–20], while a preliminary linkage map has been published for the kelp *Laminaria japonica* [21].

Experimental evidence suggests that mating type determination in heterothallic pennate diatoms is genetic [7,15], but how mating type determination is achieved is unknown. Here, we report on the construction of a sex-specific linkage map for *S. robusta* based on AFLP markers [22,23] and its use to identify the sex determining marker allele. Each AFLP marker was identified by a specific code referring to the corresponding PC and the estimated molecular size of the fragment in nucleotides as estimated by AFLP-markers were scored co-dominantly (i.e., following a 1:2:1 segregation pattern) whenever feasible, biparental markers were scored co-dominantly (i.e., following a 1:2:1 segregation pattern). They were scored dominantly (i.e., conforming to a 3:1 segregation pattern) when heterozygosity could not reliably be discriminated from homozygosity for the present AFLP markers were scored co-dominantly (i.e., following a 1:2:1 segregation pattern). 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appropriate mapping population type was set to option CP, a population resulting from a cross between two heterogeneously heterozygous and homozygous diploid parents, linkage phases originally known. Because for population type CP, the segregation type (SEG) might vary across the loci, a code indicating the segregation type has to be given. The SEG was set to <nn×np> for the MT⁺ markers, <lm×ll> for the MT⁻ markers and <hk×hk> for the biparental markers. The two characters left and right of the “×” in these codes correspond to the AFLP marker alleles of the first and second parent, respectively. Each different AFLP marker allele is represented by a different character. We first ran through a fairly wide range of logarithm of the odds (LOD) thresholds, from 2.0 to 14.0, to obtain a proper view of what might be the best grouping. In general, we decided to use the grouping obtained with a LOD score of 6.0. In a few cases, the grouping obtained at a LOD score of 8.0 and 14.0 was used. Only linkage groups containing at least three markers were considered for map construction. Maps were constructed in three rounds, each producing a linkage map. In this map-building procedure, each map was calculated by using the pairwise data of loci present on the map, with default settings (recombination frequency (REC) <0.4; LOD threshold >1). Once the well-fitting markers (causing a change in goodness of fit smaller than the threshold = 5) were positioned on the map (after two rounds), the remaining markers were forced onto the map by setting the jump threshold to zero. When the markers in the third map caused a jump in goodness of fit larger than an arbitrary threshold of 10, the second map was selected as the final map, otherwise the third map. Single markers with a segregation ratio in discordance with the flanking markers (i.e., markers showing strong segregation distortion flanked by a number of non-distorted markers) were discarded, and the map construction was repeated. A marker order was not forced on any linkage group during map construction. Recombination frequencies were converted to Kosambi centiMorgans (cM) prior to the map estimation. Linkage groups were constructed using the MT⁺ and MT⁻ markers. Biparental markers were included in both the MT⁺ and MT⁻ linkage maps. This way, homologous linkage groups were identified on the presence of identical biparental markers. Editing the linkage groups was done with the software MapChart [26].

Table 1. Statistics for the integrated MT⁺ and MT⁻ linkage maps of S. robusta.

|                | MT⁺   | MT⁻   |
|----------------|-------|-------|
| No. of linkage groups | 13    | 15    |
| No. of markers per linkage group | 8.6   | 11.6  |
| Min             | 3     | 5     |
| Max             | 25    | 37    |
| Median          | 12    | 13    |
| Mean            | 12.5  | 14.7  |
| Total           | 163   | 221   |
| Size of linkage groups (cM) |       |       |
| Min             | 11.9  | 12.1  |
| Max             | 115.3 | 134.9 |
| Median          | 87.8  | 69.1  |
| Mean            | 74.1  | 64.8  |
| Total           | 963.7 | 972.2 |
| Intermarker distance (cM) |       |       |
| Min             | 0.1   | 0     |
| Max             | 32.3  | 34.2  |
| Median          | 4.1   | 2.3   |
| Mean            | 6.4   | 4.7   |

Mapping the MT locus

The mapping of the mating type phenotype was done in two ways: 1) by including the phenotype as a single marker segregating as a MT⁺ or MT⁻ specific marker, indicated as SEX1 and SEX2, respectively, and 2) by QTL analysis using mixed models as implemented in the QTL menu in GenStat 14 [27]. Because too few homologous linkage groups are identified, QTL analysis was done for the two mating type specific linkage maps separately. In a preliminary search for QTL, we tested the association of individual marker loci with mating type every 5 cM along the genome, using the commonly known simple interval mapping (SIM) procedure. In a second step, we tested for QTL at particular positions after correcting for QTL elsewhere in the genome, as were identified in the preliminary analysis. This procedure is commonly known as composite interval mapping (CIM). The genome-wide type I error rate was set to α = 0.05. The P values calculated assume normally distributed errors, when a binomial distribution is more appropriate in the case of mating type. A previous study [28] has shown that applying the mixed model to binary traits is robust and do not result in an excess of low P values (i.e. false discoveries) as long as the minor allele frequency of the response variable and the markers is not too low.

Results

Segregation analysis and linkage mapping

A total of 54 EcoRI+MseI AFLP PCs, generating on average 8.6 markers, resulted in a total of 463 AFLP fragments segregating in the 116 F₁ progeny. In total, 162 MT⁺ and 221 MT⁻ markers were used for the construction of 13 MT⁺ and 15 MT⁻ specific linkage groups, covering 963.7 cM and 972.2 cM respectively (Figure 1 and 2). Of those marker loci, 28% displayed significantly distorted segregation ratios at the α = 0.05 significance level. As segregation distortion is a normal phenomenon in wide crosses, these markers were not a priori excluded, but evaluated after map construction. Although some larger genomic regions did not reveal any markers (e.g. 32.3 cM in the MT⁻_8 linkage group and 34.2 cM in linkage group MT⁻_9), the median inter-marker distances were relatively low (4.1 and 2.5 cM for the MT⁺ and the MT⁻ linkage maps respectively) (Table 1).

Sixty-four biparental markers were mapped to the MT⁺ and MT⁻ map separately and 20 of those markers showed cosegregation with MT⁺ and MT⁻ specific linkage groups. Five homologous maps were identified based on the presence of one or more identical biparental markers (Figure 3). No significant difference was observed between the intervals of the 20 biparental markers in the MT⁺ and MT⁻ linkage groups (paired t-test, t = 0.79; P = 0.22), suggesting that recombination frequencies do not differ much between both mating types in S. robusta.
Mapping the mating type locus

The mating type phenotype was mapped as a single marker segregating as either a MT\(^+\) (SEX1) or MT\(^-\) specific marker (SEX2). SEX1 could be assigned to the MT\(^+\)_6 linkage group, including 18 markers and spanning 115.3 cM. SEX1 is flanked by E43M124_M423.6 at 1.7 cM and E44M121_M475.8 at 5.8 cM distance. In contrast, SEX2 did not cosegregate with any of the markers of the MT\(^-\) linkage groups (highest LOD = 2).

A QTL analysis of the mating type phenotype confirmed the monogenic nature of mating type and the identification of MT\(^+\) as the heterogametic sex in *S. robusta* (Figure 4). The genome-wide significance threshold \((P = 0.05)\) for detection of QTL co-segregating with mating type was calculated as \(-\log_{10}(P) = 3.21\). The mating type phenotype mapped significantly \((-\log_{10}(P) = 198.29; E43M124M423.6)\) to a single locus located on the MT\(^+\)_6 linkage group (Figure 4a). In contrast, no significant
association was identified between the mating phenotype and MT\textsuperscript{2} specific markers. (Figure 4b).

The two homologous linkage groups MT\textsuperscript{2}_12 and MT\textsuperscript{+}_6 (Figure 3) contain six biparental markers widely spread across the two linkage groups. These identify a relatively large region of recombination between the two sex homologous chromosomes.

**Discussion**

In this study we exploit the high multiplex ratio of AFLP technology to construct the first linkage maps for a diatom species. We applied these maps to demonstrate that sex determination in the heterothallic pennate species *S. robusta* is genetic, and identify the sex determining region as a single locus.

Segregation and linkage analysis of the 463 AFLP markers scored for 116 individuals of an F\textsubscript{1} mapping population resulted in 13 MT\textsuperscript{+} and 15 MT\textsuperscript{2} specific linkage groups. The use of biparental markers, segregating in a 1:2:1 mode and scored co-dominantly, allowed the detection of five putative homologous linkage groups, including those carrying the mating type locus.

The mating type phenotype cosegregates with markers of a MT\textsuperscript{+} specific linkage group, identifying MT\textsuperscript{+} as the heterogametic sex and MT\textsuperscript{2} as the homogametic sex.

Unlike ‘classical’ sex chromosomes (like the XY chromosomes of mammals and WZ chromosomes of birds), which have only a small recombining pseudo-autosomal region (PAR) or do not recombine at all (e.g. the *Drosophila* Y chromosome), the homologous sex linkage groups in *S. robusta* appear to have a relatively large region of recombination. It is therefore likely that its X and Y chromosomes are cytologically indistinguishable (or non-heteromorphic, cf. Bergero and Charlesworth [29]). Sex chromosomes with extensive recombining PAR regions (in which both homologue chromosomes carry the same gene content) and small non-recombining regions, have been hypothesized to represent recently evolved sex chromosome systems. However, few estimates are available for the age of non-recombining regions (e.g. <2 MY ago in the papaya plant *Carica papaya*), and some data suggest that such regions may also be much older (e.g. in some bird and snake species) [29]. Studies on divergence times in the non-recombining regions of the sex chromosomes in *S. robusta* and other diatoms can contribute to our understanding of the evolution of sex chromosomes, as the diatoms, having an extensive fossil record, have a relatively well time-calibrated evolutionary record. Heterothally is to date known only in the pennate diatoms, which in the fossil record appeared in the Late Cretaceous (about 75 MY ago) [30]. All studied centric diatoms, from whom the pennate diatoms evolved, appear to be homothallic (i.e. they have no mating type

**Figure 4. QTL mapping of the mating type phenotype in 116 *S. robusta* F\textsubscript{1} progeny.** QTL analysis was done for the two mating type specific linkage maps separately. Linkage scores (plotted as \(-\log_{10}(P)\)) for MT\textsuperscript{+} (a) and MT\textsuperscript{2} (b) markers are shown according to genome position. The linkage analysis indicates that a single locus on the MT\textsuperscript{+}_6 linkage group determines the mating type in *S. robusta*. doi:10.1371/journal.pone.0060132.g004
In some cases, clones are predominantly male or female, but homothallism is still present [7]. While more studies on mating systems in centric diatoms are still needed to confirm that homothallism is the rule in centric diatoms, all available evidence so far suggests that in diatoms the evolution of heterogametic sex determination is a relatively recent event, coinciding with the transition from homothally to heterothally.

The first diatom linkage maps presented in this study will constitute an important resource for future genetic analyses in *S. robusta*. Linkage maps provide important insights into genome organization and can be used for genetic studies of traits of interest [31]. A particular advantage is that each individual progeny of the F1 mapping population can be clonally propagated and they are maintained as cryopreserved strains. A panel of immortalized F1 individuals has a number of advantages for genetic mapping identical to those of Recombinant Inbred Lines (RILs), often used in plant or rodent genetics: one needs to genotype each progeny only once and can phenotype multiple individuals from each clonal culture to reduce individual, environmental and measurement variability for multiple traits.

The linkage maps will also provide important information about the *S. robusta* genome sequence which is under construction (A. Bones & T. Brembs, pers. comm.). The completion of the genome sequence will also be the opportunity to further progress on the linkage mapping, as it will represent a source of single nucleotide polymorphisms (SNPs) and insertion/deletion (INDEL) markers for mapping. This will provide a framework to solve the position and order of scaffolds during assembly [31]. This information can be used to construct pseudo-chromosomes by concatenating adjacent supercontigs, and to carry out broad analyses of genome composition. Despite the rapidly growing amount of diatom genomic information [32], almost nothing is known about the regulation of the unique diatom life cycle. This is changing fast with the introduction of new model diatoms in which the life cycle and sexual reproduction can be reliably manipulated experimentally, including *S. robusta* [3,14]. Further characterization of the mating type locus will prove crucial for our understanding of regulation of the diatom life cycle. The identification of the *S. robusta* genomic region carrying the mating type locus in this study provides a starting point for further fine-mapping of the locus and the identification of the gene(s) and sequence polymorphism(s) underlying mating type dimorphism in *S. robusta*. In turn, this will pave the road for understanding the mechanisms underlying mating system switches to alternative reproductive modes (homothally, paedomegany, and apomixis), which are regularly observed among closely related pennate diatoms [12,13,33], and more broadly, the evolution of the MT locus in diatoms following the evolution of the penamate lineage from a homothallic centric ancestor.

### Supporting Information

**Table S1** List of primer combinations used for AFLP analysis; E: EcoRI primer with two selective bases; M: MseI primer with three selective bases, selective bases: 1,2,3,4 corresponds to A, C, G, T.

**Table S2** AFLP markers underlying the single QTL detected for the MT phenotype.

### Author Contributions

Conceived and designed the experiments: IV KS WV PV MV. Performed the experiments: IV PV. Analyzed the data: IV MV. Contributed reagents/materials/analysis tools: PV MV. Wrote the paper: IV KS WV PV MV.

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