A first-generation genetic linkage map of the European flat oyster *Ostrea edulis* (L.) based on AFLP and microsatellite markers

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Summary

This study presents the first genetic linkage map for the European flat oyster *Ostrea edulis*. Two hundred and forty-six AFLP and 20 microsatellite markers were genotyped in a three-generation pedigree comprising two grandparents, two parents and 92 progeny. Chi-square goodness-of-fit tests revealed high segregation distortion, which was significant for 32.8% of markers. Sixteen microsatellites and 235 AFLPs (170 type 1:1 AFLPs and 65 type 3:1 AFLPs) were used to build sex-specific linkage maps using CRIMAP software. The first parental map (P1) consisted of 104 markers grouped in nine linkage groups, and spanned 471.2 cM with an average spacing of 4.86 cM. The second parental map (P2) consisted of 117 markers grouped in 10 linkage groups (which equals the haploid chromosome number), and covered 450.0 cM with an average spacing of 4.21 cM. The estimated coverage of the genome was 82.4% for the P1 map and 84.2% for the P2 map. Eight linkage groups that were probably homologous between the two parents contained the same microsatellites and 3:1 AFLPs (segregating through both parents). Distorted markers were not randomly distributed across the genome and tended to cluster in a few linkage groups. Sex-specific differences in recombination rates were evident. This first-generation genetic linkage map for *O. edulis* represents a major step towards the mapping of QTL such as resistance to bonamiasis, a parasitosis that has drastically decreased populations of flat oysters since the 1960s.

Keywords amplified fragment length polymorphism, flat oyster, genetic linkage map, microsatellite, *Ostrea edulis*.

Introduction

The European flat oyster or ‘native’ oyster, *Ostrea edulis*, is endemic to the Atlantic and Mediterranean coasts of Europe. Natural populations are found in eastern North America from Maine to Rhode Island, following intentional introductions in the 1940s and 1950s (Jaziri 1990). *Ostrea edulis* exhibits interesting reproductive characteristics such as sequential protandrous hermaphroditism, with the possibility of changing sex several times in the same reproductive season, and brooding of eggs and early larvae in the mantle cavity (Yonge 1960; Le Dantec & Marteil 1976).

The flat oyster industry was of considerable economical importance in the 19th century in France and Britain (Neild 1995). Massive mortalities occurred around the turn of the 20th century from which the industry has never recovered. Oyster aquaculture production fell further from 30 000 t in 1970 to 6000 t [Food and Agriculture Organization of the United Nations (FAO) 2006] because of two parasitic diseases, martelliasis (caused by *Martelia refringens*) and bonamiasis (caused by *Bonamia ostreae*).

Since 1985, Ifremer (French Research Institute for Exploitation of the Sea) has been undertaking a selective breeding programme for resistance to bonamiasis with the main aim of producing families of oysters tolerant to the protozoan parasite *B. ostreae* (Haplosporidian protist. Carnegie et al. 2000). A similar approach was also used in Ireland (Culloty et al. 2004). In France, two improved oyster strains (S85 and S89) were produced by individual selection in mass spawning progenies (Naciri-Graven et al. 2004).
1998) and represent a valuable genetic resource for QTL mapping of this trait.

Genetic linkage maps have been established for almost all major aquaculturally important species, including tilapia (e.g. Agresti et al. 2000), catfish (e.g. Waldbieser et al. 2001), salmon (e.g. Moen et al. 2004), rainbow trout (e.g. Nichols et al. 2003), abalone (e.g. Baranski et al. 2006) and shrimp (e.g. Li et al. 2003). A few studies reported the construction of genetic maps in bivalves, including the Pacific oyster *Crassostrea gigas* (Hubert & Hedgecock 2004; Li & Guo 2004), the Eastern oyster *Crassostrea virginica* (Yu & Guo 2003), the blue mussel (Laillias et al. 2007) and the Zhikong scallop *Chlamys farreri* (Wang et al. 2004, 2005; Li et al. 2005). QTL have been mapped in a few shellfish species, including disease resistance in *C. virginica* (Yu & Guo 2006) and production traits in the Kuruma prawn (Li et al. 2006a). A genetic linkage map for the flat oyster is a first step towards the identification of QTL for resistance to bonamiasis and the eventual development of marker-assisted selection (MAS) in this species.

**Materials and methods**

**Mapping family**

The first stage of the selective breeding programme initiated by Ifremer in 1985 consisted of the production of two improved oyster strains (S85 and S89) by mass selection (Naciri-Graven et al. 1998). Selection was applied both through inoculation tests (Mialhe et al. 1988) in an experimental hatchery and by field testing in natural conditions. In parallel to the selection programme, several generations of inbred lines have been produced since 1995. These inbred lines were initiated by crossing the selected oyster strain S89 and a wild-type oyster, followed by successive full-sib matings. A sixth-generation inbred line, OELL2000-set2, has had zero mortality from bonamiasis in the field since 2000.

The mapping family used in this study was initiated in 2003 by crossing a wild-type oyster (W102) and an oyster from the inbred line OELL2000-set2 (L002-53). Two full-sibs from this F1 family were then crossed to make the mapping family (OEF2.04.63). The mapping family consisted of two grandparents (F0; L002-53 and W102), two parents (F1; 23–31 and 23–32) and 92 progeny (F2). The mapping family was sampled when the progeny were about 15 months old.

In order to achieve bi-parental crosses, oysters were held in pairs in small aquaria. Each aquarium was individually supplied with filtered sea water and the outlet pipe of each aquarium was placed above a 100-μm mesh sieve for the collection of late larvae. It is not possible to non-destructively identify female oysters even when they are brooding, so the respective sexes of the two F1 parents were not determined. The F1 parent 23–31 was referred to as ‘Parent 1’ or ‘P1’ and the F1 parent 23–32 as ‘Parent 2’ or ‘P2’.

**Genotyping**

DNA was extracted from gill tissue using a standard chloroform extraction followed by purification with the Wizard® DNA Clean-Up System (Promega) (Wilding et al. 2001). Quality and concentration of each DNA sample was assessed using a spectrophotometer and by running a small amount on a 2% agarose gel.

Twenty microsatellite markers selected from those developed by Naciri et al. (1995), Launey (1998), Morgan et al. (2000), Morgan & Rogers (2001), Sobolewska et al. (2001) and Launey et al. (2002) were amplified by PCR according to the authors’ protocols. Markers informative in the parents were genotyped across the full family.

AFLP analysis was performed using a modified version of Vos et al. (1995); the protocol followed Wilding et al. (2001), but digestion and ligation were achieved in the same mix by incubating for 16 h at 16 °C. Sixty AFLP primer pairs were genotyped in the mapping family (Table S1). Electrophoresis and data collection were carried out on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Electrophoresis parameters were set at injection for 15 s at 15 kV, and running at 60 °C for 25 min at 15 kV with POP4 polymer. Data were analysed with GENEMAPPER® software version 3.7, and individuals were scored for the presence [A] or absence [a] of the amplified AFLP fragment (peak). A peak-absent marker phenotype was assumed to be the homozygote genotype aa.

**Distortion of segregation ratios**

Segregation distortion analysis was performed using the chi-square goodness-of-fit statistical test between the F1 parents and the F2 progeny. For the codominant microsatellites, three types of segregation could be observed depending on the number of alleles present in the two parents: genotypic distributions of 1:1:1:1; 1:2:1 and 1:1. For the dominant AFLP markers, there were only two genotypic classes: presence or absence of the peak. Two types of segregation could be observed, either 1:1 (when only one of the two parents exhibited the peak) or 3:1 (when both parents exhibited the peak). Inference of AFLP genotypes from the phenotype (presence or absence of peak) allowed determination of the following AFLP transmissions:

- Type I, for which only one F0 parent and one F1 parent exhibited the peak. Each phenotype could be assigned to a genotype (Aa for presence of the peak or aa for absence of the peak) and the grandparental and parental origins of AFLP alleles could be tracked without ambiguity (1:1 segregation type).
- Type II, for which both grandparents were Aa and only one F1 parent exhibited the peak. Each phenotype could be assigned to a genotype (Aa or aa) but it was not possible to assign the grandparental origin of the A allele (1:1 segregation type).

- Type III, for which only one F₀ parent and the two F₁ parents exhibited the peak. The presence of a peak in the F₂ progeny led to an ambiguous genotype A₁ (either AA or Aa), but the grandparental origin of the A allele could be tracked (3:1 segregation type).

Linkage analysis

CRIMAP software (Green et al. 1990) was used for the construction of genetic linkage maps. First, the Two-Point option was used to estimate recombination frequencies between each pair of markers for each of the two F₁ parents, at a LOD score ≥ 3.0. These two-point data were used to construct parent-specific linkage groups. The second step consisted of ordering markers within each linkage group using the Build command, with sequential incorporation of loci starting with the most informative pair of markers. The Fixed and All commands were used to add the unplaced markers after Build into the map, by decreasing order of informativeness. After the addition of a new marker to a sequence of ordered markers, the new order was tested against alternative order using the Flips command. Markers that led to an ambiguous map position (i.e., markers that had two or more alternative map positions with a small difference in their likelihood) were discarded from further analysis. Finally, the Chrompic command was used to display the number and location of recombinations on each chromosome, highlighting candidate data errors.

The mapping of 20 microsatellite markers and type III AFLPs (segregating through both Parent 1 and Parent 2) in the two parental genetic linkage maps P₁ and P₂ allowed the identification of probable homology groups. Homology groups were assumed when at least two markers (microsatellites and/or AFLPs) were linked in two linkage groups (P₁ and P₂) and when several markers in the same linkage group were common to both the P₁ and P₂ maps. Indeed, some common markers were linked but could not be assigned to the two parental maps because they had ambiguous map positions and were therefore discarded.

Genome size and coverage

Average marker spacing of each map was calculated by dividing the total length of the map by the number of intervals. The average marker spacing for each linkage group was calculated by dividing the length of each linkage group by the number of intervals on that linkage group. The expected length of the genome was estimated using method 4 of Chakravarti et al. (1991). Genome coverage estimates were determined by dividing the observed genome length by the expected length of the genome.

Recombination frequency

Differences in recombination frequencies between the two parents were estimated using G-tests of independence that compared parental and recombinant genotypes for each parent for each pair of linked markers (with LOD score > 3.0).

Results

Segregation distortion

High segregation distortion was evident in the mapping family. Only 25% of the microsatellites (four out of 16 informative markers: Oe1/47, Oe3/37, Oedu.HA21 and Oedu.B11) appeared to segregate according to Mendelian rules. Sixty-one per cent of the 1:1 AFLPs (107 out of 175 markers) and 92% of the 3:1 AFLPs (65 out of 71 markers) exhibited Mendelian segregation. Overall, 69% of the AFLP markers (172 out of 246 markers) were considered to have Mendelian inheritance. The high percentage of Mendelian 3:1 AFLPs compared with the 1:1 AFLPs was probably due to the fact that selection of 3:1 markers was based on a preliminary scoring of 48 F₂ individuals after which highly distorted 3:1 AFLPs were discarded. In total, 16 microsatellites and 235 AFLPs (170 type 1:1 AFLPs and 65 Mendelian type 3:1 AFLPs) for a total of 251 markers were included in the final linkage analysis. Distorted microsatellites and 1:1 AFLPs were included in the analysis after mapping the non-distorted markers.

Parent-specific linkage maps

The P₁ genetic linkage map was based on 16 microsatellites and 145 AFLPs segregating in this parent. The AFLPs consisted of 71 markers of type I (31 from L002-53, 40 from W102), nine of type II and 65 of type III (37 from L002-53, 28 from W102). The resulting map consisted of 104 markers (64.6% of available markers), comprising 14 microsatellites (87.5%), 62 type I AFLPs (87.3%), seven type II AFLPs (77.8%) and 21 type III AFLPs (32.3%). Nine linkage groups were set up for the P₁ map covering 471.2 cM (Fig. 1). The sizes of the linkage groups ranged from 23.6 to 95.8 cM. The number of markers per linkage group varied from 4 to 22. The average distance between two loci ranged from 3.16 cM (P₁₃) to 10.1 cM (P₁₈), with an average spacing of 4.86 cM. The largest interval varied from 9.7 cM (P₁₃) to 35.3 cM (P₁₄) (Table 1). The observed map length was 471.2 cM for the P₁ map, and the estimated genome length was 571.7 cM. The observed coverage was therefore 82.4% for the P₁ map.

The P₂ genetic linkage map was based on 16 microsatellites and 154 AFLPs segregating in this parent. The
Genetic linkage map of Ostrea edulis

Figure 1  Microsatellite and AFLP-based linkage maps of the flat oyster Ostrea edulis in the mapping family OE.F2.04.63: P1 (23–31) and P2 (23–32) maps obtained with CRIMAP. AFLP markers are labelled with the primer pair name followed by the letter 'f' (for fragment) and a three-digit fragment size in base pairs. Markers are indicated on the right and absolute positions on the left (in Kosambi cM). The segregation type (I, II or III; see text) and the direction of the segregation distortion: towards a deficit (−) or excess (+) of aa homozygotes are included on the right of the AFLP locus. Lines between P1 and P2 groups indicate homologous positions, with common markers underlined.
AFLPs consisted of 84 markers of type I (38 from L002-53, 46 from W102), five of type II and 65 of type III (37 from L002-53, 28 from W102). The resulting map consisted of 117 markers (76.0% of available markers), comprising 14 microsatellites (87.5%), 76 type I AFLPs (90.5%), five type II AFLPs (100%) and 22 type III AFLPs (33.8%). Ten linkage groups were set up for the P2 map, covering 450.0 cM (Fig. 1). The sizes of the linkage groups ranged from 11.9 to 77.7 cM. The number of markers per linkage group varied from 2 to 24. The average distance between two loci ranged from 1.34 cM (P2_11) to 26.1 cM (P2_10), with an average spacing of 4.21 cM. The largest interval varied from 10.5 cM (P2_11) to 26.1 cM (P2_10) (Table 1). For the P2 map, the observed map was 450.0 cM and the estimated genome length 575.8 cM. The observed coverage was 84.2% for the P2 map.

Eight probable homology groups were identified. No homology group was found for P1_9, P2_10 and P2_11. Two pairs of homology groups (P1_2 and P2_2; P1_8 and P2_8) were found based on linkage of several markers that were mapped in only one of the two parental maps: P1_2 and P2_2 have four common markers that could be mapped only in P2_2; P1_8 and P2_8 have two common markers that could be mapped only in P2_8 (Table 1 and Fig. 1). Comparison of orders of markers between the two parental maps was possible for the six homology groups in which common markers were mapped in both P1 and P2 maps. Marker order seemed conserved for most homology groups. The greatest discrepancy occurred for group 1 where five microsatellites were not in the same order in the two maps. The lengths of homology groups 3 and 5 were similar between the P1 and P2 maps but in most cases there was a discrepancy in homology group lengths between the two maps, particularly for group 1 and group 6 (Fig. 1).

Distorted AFLP markers showed non-random distribution or clustering in both genetic maps (P1 and P2). In the P1 map, the 30 mapped distorted AFLPs (type 1:1) were mainly located on four linkage groups, P1_2 (containing 12 aa homozygote deficiency markers in a 35-cM segment), P1_5 (containing three aa homozygote deficiency markers and one aa homozygote excess marker), P1_6 (containing six aa homozygote deficiency markers in a 38-cM segment) and P1_9 (containing two markers with aa homozygote deficiency and two with aa homozygote excess). In addition, four other groups contained each only one distorted marker showing aa homozygote deficiency: P1_1, P1_4, P1_7 and P1_8. In the P2 map, the 26 mapped distorted AFLPs were concentrated on three linkage groups: P2_2_1 (containing nine aa homozygote deficiency markers and one aa homozygote excess marker), P2_11 (containing five markers with aa homozygote deficiency and five with aa homozygote excess in a 5-cM segment) and P2_7 (containing four aa homozygote deficiency markers in a 16-cM segment). In addition, two other groups, P2_2 and P2_6, each contained one distorted marker showing aa homozygote deficiency (Fig. 1).

**Distribution of markers**

The assumption of a random distribution of AFLP markers in the genome was tested by Spearman correlation coefficients and chi-squared test for departure from a Poisson distribution following Barreneche et al. (1998). Spearman correlation coefficients (r) between genetic length and number of markers per group were 0.85 for Parent 1 (u_1 = 2.40, P < 0.05) and 0.61 (u_2 = 1.84; P > 0.05) for Parent 2. Therefore, AFLP markers were generally randomly distributed in the linkage groups of the P1 map but not in

<table>
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<tr>
<th>Linkage group</th>
<th>Common markers</th>
<th>Length (cM)</th>
<th>No. markers</th>
<th>Marker spacing (cM)</th>
<th>Largest interval (cM)</th>
<th>Parent 1</th>
<th>Length (cM)</th>
<th>No. markers</th>
<th>Marker spacing (cM)</th>
<th>Largest interval (cM)</th>
<th>Parent 2</th>
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<td>21</td>
<td>4.79</td>
<td>22.5</td>
<td>65.4</td>
<td>17</td>
<td>4.09</td>
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<td>50.7</td>
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<td>4 (in P2)</td>
<td>72.1</td>
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<td>4.81</td>
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<td>117</td>
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<td>26.1</td>
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In brackets are the number of common markers that were mapped in both P1 and P2 maps (some common markers were linked to a group but could not be mapped).
the P2 map because of significant clustering of markers. Observed and expected distributions of AFLPs were compared for 20-cM intervals in both the P1 and P2 maps. The mean of the Poisson distribution was 4.2 for P1 and 4.95 for P2. No significant departure from the Poisson distribution was observed for Parent 1 ($\chi^2 = 3.1, 6$ d.f., $P = 0.796$). However, this goodness-of-fit test was highly significant for Parent 2 ($\chi^2 = 81.04, 13$ d.f., $P = 0.000$), mostly due to three intervals of 20 cM containing only one marker (large interval gaps remained to be filled in P2_4, P2_6 and P2_10) and to one interval of 20 cM containing 14 markers (high clustering in P2_11).

Parent-specific recombination differences

Differences in recombination frequencies were observed between the two parents. Eighty-six pairs of markers were segregating in both parents (17 pairs of microsatellites and 69 pairs of microsatellites/AFLPs). Forty-four pairs of markers showed statistically different recombination frequencies between Parent 1 and Parent 2; 12 of these were associated with higher recombination in Parent 2 and 32 with higher recombination in Parent 1 (Fig. 2).

Discussion

Mapping family and experimental design

The mapping family consisted of a three-generation pedigree (grandparents, parents and offspring) that did not come from truly inbred lines (homozygous for all loci), but from a cross between a sixth-generation inbred line and a wild oyster. This mating scheme is unusual for a mapping family in a shellfish species. Indeed, the classical mating schemes in experimental populations where inbred lines are available generally involve the analysis of either backcross or F2 families (Shimoda et al. 2000; Sakamoto et al. 2000; Li & Guo 2004) or F2 families (Shimoda et al. 1999; Li et al. 2003). Other mapping panels consisted of two-generation pedigrees (parents and offspring) (Coimbra et al. 2003, Lallias et al. 2007). However, when studying natural populations, or when inbred lines are not available, individuals can be taken from the population, genotyped and mated in pairs to yield a number of full-sib families. In a particular family, any pair of segregating loci will represent either an F2 (if both parents are heterozygous for the pair of markers) or a backcross (if only one parent is heterozygous whereas the other is homozygous) (Kearsey & Pooni 1998). This strategy has been used in several studies (Waldbieser et al. 2001; Yu & Guo 2003; Wang et al. 2005; Li et al. 2006b; Lallias et al. 2007) and is the most commonly used experimental design in shellfish species.

However, in the context of QTL mapping, a three-generation pedigree was chosen for our mapping family in O. edulis. This experimental design proved to be efficient for the mapping of microsatellites (87.5% mapped in both parents) and type I AFLPs (87.3% mapped in P1 and 90.5% mapped in P2). However, as expected according to the informativeness of the markers (Ritter et al. 1990), the mapping of type III AFLPs (3:1 segregation) in an F2-type family was less powerful because only 32.3% and 33.8% were mapped in the two parental maps.

Segregation distortion

High segregation distortion was evident in our mapping family of O. edulis. Overall, 32.8% of the markers were distorted with microsatellites being commonly so (75%) and AFLPs much less so (31%). The range of segregation distortion reported in this study was similar or slightly higher than that reported in another oyster species, C. gigas: 31% with allozymes (McGoldrick & Hedgecock 1997), 20.9% with microsatellites (Launey & Hedgecock 2001) and 26.9% with AFLPs (Li & Guo 2004). In our study, the high proportion (85.1%) of distorted AFLP markers that showed an aa homozygote deficiency could be explained by a high genetic load that has previously been reported in bivalves (McGoldrick & Hedgecock 1997; Bierne et al. 1998; Launey & Hedgecock 2001). The mapping family came from crossing into a selected oyster strain that had been through a strong population bottleneck with a small effective number of breeders (Launey et al. 2001). In addition, the mapping family originated from six generations of full-sib matings that would certainly have undergone some inbreeding depression. Therefore, assuming that purging of deleterious genes by full-sib crosses was not complete by the sixth generation, it is probable that the high segregation distortion observed was because of linkage of markers with lethal or deleterious genes in the recessive state.

The mapping of distorted markers may help understand the distribution of deleterious recessive genes in the genome.
O. edulis was good, above 82%, and compared favourably to any flat oyster species. The genome coverage achieved in this species should be added to the maps for a better coverage of the genome. This was confirmed by the fact that only eight probable homology groups were found, and that no clear homology could be found for three linkage groups, P1_9, P2_10 and P2_11. Finally, some of the linkage groups consisted of only two markers or spanned a small genetic distance (<20 cM). Therefore, these groups may in fact belong to the same chromosome and may coalesce by adding more markers.

Recombination differences between the sexes
Our study reported higher recombination rates in the Parent 1 with 32 significant pairwise recombination rate differences (out of 44) compared with Parent 2 (12 out of 44 significant pairwise comparisons) (Fig. 2). Unfortunately, because of the brooding behaviour of O. edulis, the sex of our individual F1 oyster parents could not be determined. However, large sex-specific differences in recombination rates have been reported in several studies. Higher recombination rates in females were found in rainbow trout (Sakamoto et al. 2000), channel catfish (Waldbieser et al. 2001), zebrafish (Knapiak et al. 1998), C. virginica (Yu & Guo 2003), C. gigas (Hubert & Hedgecock 2004) and Penaeus monodon (Wilson et al. 2002). Therefore, these potential sex-specific differences in recombination rates in O. edulis should be confirmed and investigated further by mapping more markers.

Future uses
The development of genetic linkage maps is particularly useful for the mapping of QTL and for MAS. Several studies have highlighted the potential for MAS in breeding programmes in fisheries (Ward et al. 2000; Perry et al. 2001; Liu & Cordes 2004). MAS has a huge potential in aquaculture breeding programmes, especially for traits difficult to phenotype, but so far no successfully applied MAS has been reported in fish or shellfish species. Although disease resistance generally seems to have a low heritability in some species (Gjedrem 2000), it is nevertheless an ideal trait for the application of MAS, because of the economic significance of high survival in aquaculture. Moreover, MAS would reduce the time of selection between generations. Several studies have reported the location of QTL for disease resistance in rainbow trout based on the classical approach for QTL mapping using interval mapping, the ANOVA-based approach or bulk segregant analysis (Palti et al. 1999; Ozaki et al. 2001; Rodriguez et al. 2004). Disease resistance is of particular interest for the flat oyster, which has suffered such a huge decline from parasitic diseases, and MAS for disease resistance could be an important tool in the regeneration of oyster aquaculture. The O. edulis genetic map described here represents a first step towards the search for QTL in this species.
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References


Supplementary material

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Table S1 Primer pairs used for scoring AFLPs.

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