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Genomic sequences and genetic differentiation at associated tandem repeat markers in growth hormone, somatolactin and insulin-like growth factor-1 genes of the sea bass, *Dicentrarchus labrax*

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Abstract – The completion of genomic sequences of physiologically important genes frequently reveals non-coding genetic elements such as tandem repeats (micro- and minisatellites) that are often more polymorphic than nearby coding sequences. We obtained the complete genomic sequences of three hormone genes in sea bass *Dicentrarchus labrax*: growth hormone (*dlGH*), somatolactin (*dlSL*) and insulin-like growth factor-1 (*dlIGF-1*), including 5′- and 3′-untranslated regions. Mini- and microsatellites were discovered in both flanking and intron regions. Some were partially conserved across Perciformes. To assess the usefulness and relevance of these gene-associated markers for understanding population structure, an investigation was made on genetic diversity and differentiation at four of them in (*i*) five wild populations from the North Sea, the Bay of Biscay and the Western Mediterranean, and (*ii*) two samples of hatchery-bred individuals from a freshwater-acclimation experiment. Gene and allelic diversities were lower in cultured individuals than in wild ones. Significant genetic differentiation was demonstrated between Bay of Biscay + North Sea and Mediterranean populations ($F_{st} > 0.06$, $p < 0.001$), primarily due to *dlGH*-associated markers. Significant genetic differentiation was also detected among the Atlantic and North Sea samples, but restricted to the locus associated with *dlSL*. Significant genetic differentiation was also found among experimental individuals before and after a salinity challenge ($F_{st} \approx 0.05$, $p < 0.001$), but was due to *dlSL* and *dlIGF-1* loci. Gene-associated markers proved to be more efficient than formerly used anonymous microsatellite markers in providing a clear picture of genetic differentiation.

Key words: Growth hormone / Somatolactin / Insulin-like growth factor-1 / Non-coding regions / European sea bass / Microsatellites / Minisatellites

Résumé – Connaître les séquences génomiques de gènes d'importance physiologique permet de déceler des éléments génétiques non-codants tels que des répétitions en tandem (micro - et minisatellites), souvent porteurs de davantage de polymorphismes que les séquences codantes voisines. Des séquences génomiques complètes ainsi que celles de leurs régions flanquantes en 5′ et 3′ sont établies pour trois gènes codants pour des hormones chez le bar, *Dicentrarchus labrax* : l'hormone de croissance (*dlGH*), la somatolactine (*dlSL*) et le facteur de croissance apparenté à l'insuline (*dlIGF-1*). Ainsi, un mini- et des microsatellites partiellement conservés chez les Perciformes ont été trouvés. Pour évaluer la pertinence de ces marqueurs pour des études de structure génétique des populations, leur diversité et leur différenciation génétiques sont examinées chez (i) cinq populations sauvages de la mer du Nord, du golfe de Gascogne et de la Méditerranée occidentale, ainsi que dans (ii) deux échantillons d'individus d'élevage tirés d'une expérience d'acclimatation à l'eau douce. L'hétérozygotie et la diversité allélique sont inférieures chez les individus d'élevage, comparées à celles d'individus sauvages. Une différenciation génétique significative est établie entre populations de Gascogne + mer du Nord et les populations de la Méditerranée ($F_{st} > 0{,}06$; $p < 0{,}001$), principalement en raison des marqueurs associés à *dlGH*. Une différenciation génétique significative est également mise en évidence entre les échantillons de Gascogne et de la mer du Nord, mais uniquement

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pour le locus associé à *dlSL*. Enfin, pour les marqueurs associés à *dlSL* et *dlIGF-1*, une différenciation génétique significative est mise en évidence entre individus expérimentaux échantillonnés avant et après la dessalure ($F_{st} \approx 0,05$; $p < 0,001$). Ces marqueurs associés à des gènes se sont avérés être plus efficaces que les marqueurs microsatellites anonymes préalablement utilisés, fournissant dans chaque cas une image claire de la différenciation génétique entre échantillons/populations.

1 Introduction

Over the last two decades, population genetics of marine fish species has mostly aimed to develop anonymous markers, in particular short sequence repeat loci (SSR, also known as microsatellites). These markers have served to investigate patterns of gene diversity and rates of neutral evolution, to describe spatial and temporal genetic structure of species, and to derive guidelines for species management. Extensive knowledge of the distribution of such markers across the genome was generally not sought, as most polymorphic SSRs in animals are known to be located in genomic regions without known functions (e.g. Hancock 1999). A recent renewed interest in studies aiming to link physiological, ecological and/or adaptive performance with allozymic genotypes (e.g. Koehn et al. 1980; Di Michele and Powers 1982) has led to a more focused effort to describe functional genomic variation, specifically targeting genetic variation in coding regions (e.g., exons: Zheng et al. 2009; expressed sequence tags [EST]: Vasemägi et al. 2005; Bouck and Vision 2007), as well as in non-coding regions of candidate genes (e.g. introns: 5′- or 3′-untranslated regions [UTR] promoters). Indeed, while such polymorphisms might also largely be neutral, evidence has accumulated about the importance of genetic variation for adaptation in some of these non-coding regions (e.g. Kashi and Soller 1999; Li et al. 2004; Wray 2007). In some cases, extensive conservation of coding sequences at candidate genes leaves polymorphisms in non-coding regions as the only targets for evolutionary studies (for growth hormone, see Ryynänen and Primmer 2004) In these non-coding regions, molecular polymorphisms such as SNPs (single-nucleotide polymorphisms), indels (insertion-deletion) and SSRs may modulate gene expression, and therefore be directly or indirectly related to phenotypic variation (Fig. 1). Thus, they may influence individual performance and fitness, and so participate in differential adaptation of organisms to their environment (e.g. Feder and Mitchell-Olds 2003; Dalziel et al. 2009; Nielsen et al. 2009a; see Fig. 1). Genotype-phenotype relationships that implicate candidate genes are still scarce among fish species. Nevertheless, they are becoming more frequently documented (Pradet-Balade et al. 1998; Schulte et al. 2000; Streelman and Kocher 2002; Tao and Boulding 2003; Almuly et al. 2005, 2008; Fromme et al. 2009; Li et al. 2009; Zhang et al. 2009; Blel et al. 2010). As noted by Nielsen et al. (2009a), the candidate gene approach necessitates the choice of the "right" candidates (i.e. genes whose variant distribution reflects biologically important adaptive variation), which are certainly numerous in fish because they are in such intimate contact with their surrounding medium, and thus sensitive to environmental factors such as temperature, oxygen levels, and salinity (see also Cossins and Crawford 2005).

The European sea bass (*Dicentrarchus labrax*; Perciforms, Moronidae) is a demersal fish distributed in the Eastern Atlantic from Mauritania to Norway and throughout the Mediterranean and Black sea, exploiting natural habitats of widely variable salinity (0.5 to 40 psu; Kelley 1988; Dufour et al. 2009). It is one of the primary species in European marine aquaculture (Pickett and Pawson 1994; Chatain and Chavanne 2009), and is now reared at an industrial scale (>80 000 t y^{-1}; Chatain and Chavanne 2009). Population genetic structure of wild sea bass is very well described, based on allozymic, anonymous SSR and mitochondrial DNA markers (review in Chatain and Chavanne 2009), and patterns of sea bass genetic differentiation are a paradigm for many other marine species (Patarnello et al. 2007). Primarily due to its value to fish farming, new genomic resources have accumulated for sea bass (e.g. Volckaert et al. 2008; Kuhl et al. 2010a,b). More than three hundred SSR loci have been identified in the *D. labrax* genome (Volckaert et al. 2008), and a subset of them has been arranged in linkage groups reflecting their putative co-distribution on the chromosomes (Chistiakhov et al. 2005, 2008). Nevertheless, as most of these SSR were extracted from enriched genomic libraries, they have primarily been detected because of their repeated sequences rather than any putative association with genes. Only a few of them have been located near recognised coding sequences (31 SSRs over 190 [16.3%]; Chistiakhov et al. 2008). Furthermore, the physiological role of the relevant genes is often very poorly documented in fish, making these markers inadequate for the investigation of functional genotype-phenotype relationships.

In this study, we adopted a candidate gene approach to identify genetic markers at three hormone genes in *D. labrax*: growth hormone (*GH*), somatolactin (*SL*) and insulin-like growth factor 1 (*IGF-1*). These genes are interesting candidates because of the large existing literature on the direct or indirect physiological importance of these multifunctional hormones in osmoregulation (Sakamoto and Hirano 1993; Mancera and McCormick 1998a, 2007; Zhu and Thomas 1998; Taniyama et al. 1999; Sakamoto and McCormick 2006; Deane and Woo 2009). Furthermore, the *GH* and *SL* genes have been demonstrated to vary their expression in response to salinity variations experienced by sea bass (Varsamos et al. 2006). The involvement of GH and IGF-1 in somatic growth is also clearly established (the GH-IGF-1 system; review in Reinecke 2010), making these genes especially important for cultured species (De-Santis and Jerry 2007). A functional role for genetic variability in influencing levels of gene expression and further growth has only been reported for markers associated with GH (Almuly et al. 2005, 2008; Zhang et al. 2009) and IGF-1 (Li et al. 2009) in cultured fishes. Therefore, screening these candidate genes for polymorphisms reflecting local adaptation or artificial selection is of particular interest for population genetics and aquaculture. We first characterised the genomic

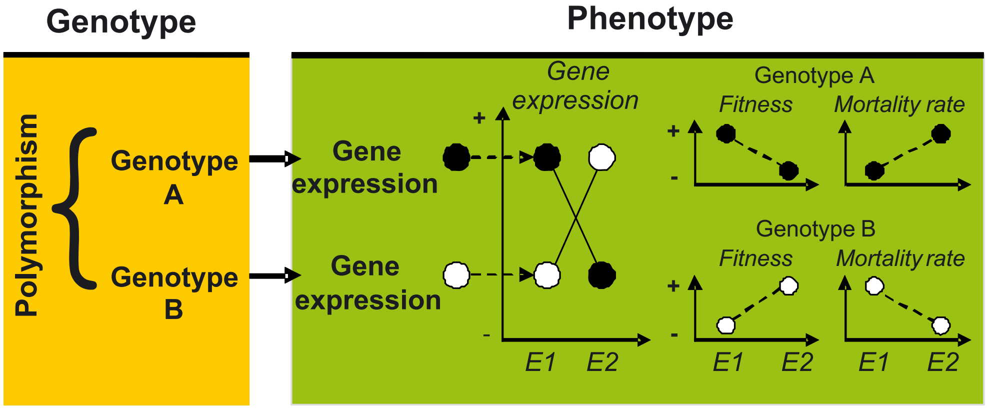

Fig. 1. Schematic representation of the genotype-phenotype relationship. Polymorphisms occurring in non-coding (this paper) or coding regions of genes might induce environmentally distinct patterns of gene expression, which depend on the genotype in question. In distinct environments (*E*), levels of gene expression associated with each genotype would enhance the performance and, therefore, the fitness of a given genotype, which would then exhibit a lower mortality rate.

sequence of these genes, then assessed the polymorphism of some tandem repeat markers (micro- and minisatellites) located in either their 5′ non-coding region or introns, as well as the conservation of these markers in other teleost species. We finally estimated genetic diversity for these markers in wild and cultured populations. For five wild populations, we assessed their respective contribution to the partitioning of sea bass among the North Sea, Atlantic (Bay of Biscay), and Western Mediterranean. The experimental population consisted of hatchery-bred individuals randomly sampled before and after a salinity challenge to evaluate selective mortality due to freshwater acclimation.

2 Materials and methods

2.1 Genomic sequence completion

GH, *SL* and *IGF-1* mRNA sequences available in NCBI (respectively X65716, AJ277390 and AY800248; http://www.ncbi.nlm.nih.gov/) were used to query the genomic scaffolds from the sea bass whole genome sequencing project in progress (Kuhl et al. 2010a). The resulting assemblies were completed for all three genes by Sanger sequencing on ABI Prism 3130xl Genetic Analyser, using primers reported in supplementary material (Annexe 1). The final genomic sequences of the *GH*, *SL* and *IGF-1* genes thus encompassed all the coding sequences and the 5′- and 3′-UTRs. Other genomic sequences of *GH*, *SL* and *IGF-1* available for teleosts were extracted from the NCBI public database (www.ncbi.nlm.nih.gov). For *GH*, cross-species amplifications were performed for other Moronidae (*Dicentrarchus punctatus* and the four *Morone* spp. composing this family) using sea bass primers, and sequenced whenever PCR products were obtained. Multispecies alignments were constructed using BioEdit (Hall 1999) to assess the conservation of homologous tandem repeat loci.

2.2 Tandem repeat polymorphism analysis

For all three genes, the detection of sequence repeats was done using Tandem Repeat Finder (Benson 1999). Once identified in all three genes, a polymorphism survey was performed on populations of distinct origin: Bay of Biscay (Atlantic; $n = 30$, used by Fritsch et al. 2007), Doel (North Sea; $n = 32$; 51°18′47″ N, 4°16′35″ E), Zeebrugge (North Sea; $n = 32$; 51°29′00″ N, 2°70′00″ E), La Goulette (Western Mediterranean, Tunisia, $n = 28$, used by Bahri-Sfar et al. 2000); Sète (Western Mediterranean, France, $n = 38$, used by Guinand et al. 2008). The Mediterranean Sea and the Atlantic-North Sea group correspond to two differentiated populations already recognised in this species (e.g. Naciri et al. 1999).

Using the progeny of broodstock fishes maintained at the *Ecloserie marine de Gravelines* (France), we further acclimated this progeny ($n = 800$) to fresh water according to the transfer protocol described in Nebel et al. (2005). This broodstock had not undergone any selective breeding. Thirty individuals from this progeny were randomly sampled before (June) and after (late September) this salinity acclimation to assess possible shifts in allele frequencies, possibly reflecting adaptive differentiation. As already reported in sea bass, salinity challenges cause differential mortality in fresh- and seawater individuals (e.g. Chervinski 1974; Allegrucci et al. 1994; Marino et al. 1994). In this study, the mortality recorded in September was 53% for freshwater-challenged individuals vs. 22% for seawater individuals (mortality still occurs in seawater due, for example, to differential access to food and cannibalistic behaviour).

PCR (polymerase chain reaction) conditions and primers for each SSR are described in Annexe 2. Genotyping of individuals was performed by allele sizing on an ABI Prism 3130xl Genetic Analyser (Applied Biosystems), using a labelled forward primer, and two different size standards (GeneScan^{TM} 1200 LIZ®, Applied Biosystems; Internal Lane Standard 600, Promega). For each fish group, unbiased heterozygosity (H, Nei 1987), allelic richness (A), departure from Hardy-Weinberg equilibrium (HWE; as measured by F_{is}; Weir and Cockerham 1984), and linkage disequilibrium

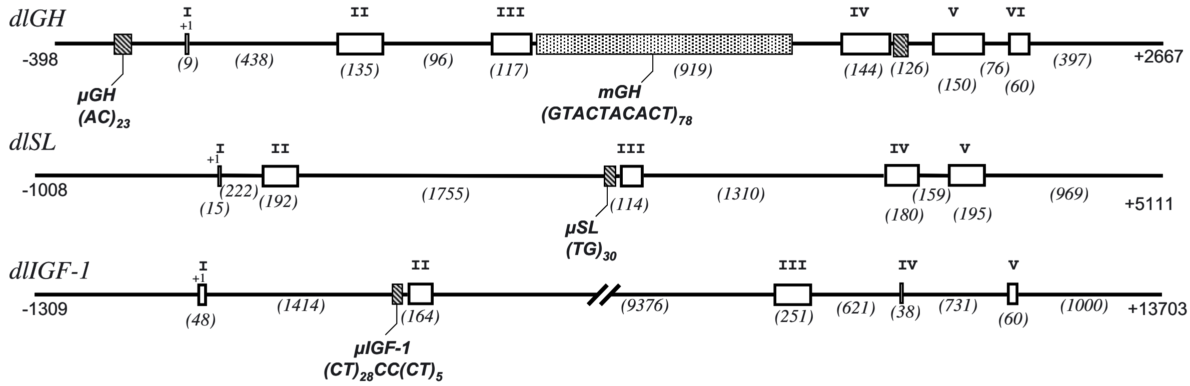

Fig. 2. Synthetic representation of the structure of *dlGH*, *dlSL* and *dlIGF-1* (GenBank accession numbers: GQ918491, GQ924782, GQ924783, respectively). Open boxes: exons; black lines: non-coding regions, i.e. introns, 5′- and 3′-flanking regions. Size (in base pairs) of each intron and exon is indicated in brackets. The lengths of the 3′-flanking regions of each gene were inferred from the position of the poly-A signal in the transcript. A MAUI element (not reported) was detected in the 5′-flanking sequence of dlGH (–1338 bp; –398 bp), and we limited representation of dlGH at this latter limit. dlIGF-1 has been interrupted to fit the figure, but the length of intron 2 is indicated. SSRs detected in this study are indicated by dashed and dotted boxes for micro- and minisatellites, respectively. SSR loci used for the genetic diversity study based wild and cultured fishes (Annexe 2) are indicated in bold.

(LD; Weir 1979) were computed using Genetix v4.05 (http://www.univ-montp2.fr/~genetix/). Genetic differentiation among samples was evaluated using F_{st} (Weir and Cockerham 1984). Tests aiming at detecting significant departures from HWE, significant genetic differentiation among samples, and significant LD were carried out by permutations (1000 bootstrap replicates) in Genetix v4.05. Corrections for multiple tests were considered when necessary.

3 Results

3.1 Gene organisation

Complete genomic sequences of each gene were deposited in the Genbank data base under accession numbers GQ918491 (*dlGH*), GQ924782 (*dlSL*), and GQ924783 (*dlIGF-1*). Schematic representations of these genes are reported in Figure 2, including the sizes (in bp) of each exon and intron, as well as the flanking sequences. These sequences complement the previously deposited cDNA (coding DNA) sequences available for sea bass. Coding sequences established in this study were consistent with previously deposited cDNA sequences for *dlGH* (GenBank accession number: X65716; Doliana et al. 1992), and *dlIGF-1* (AY800248; Company et al. 2000). For *dlSL*, our sequence completes the coding sequence of the gene by adding the first exon (Fig. 2) to the previously published mRNA (AJ277390; Terova et al. 2007). The genomic sequences of the genes span over 3125 bp (base pairs) for *GH*, 6119 bp for *SL*, and 15 012 bp for *IGF-1* (Fig. 2), and gene organisation matches canonical structures reported for these genes in numerous teleosts, primarily in Perciformes (*GH*: 6 exons/5 introns; *SL* and *IGF-1*: 5 exons/4 introns). For *dlGH*, it should be further noted that the 5′ sequence included a MAUI element (i.e. a non-LTR retrotransposon) localised only 400 bp upstream of the start codon. This MAUI element was not presented in our sequence shown in Figure 2, but was included in the deposited sequence.

Cross-species PCR within the moronids were performed for genomic sequences, from intron 1 to the 3′ end of the *GH* gene. Possibly because of more sequence divergence and presence of the MAUI element, we failed to amplify upstream sequences of *D. punctatus* and *Morone* spp., including the first exon and the 5′-UTR region of the gene. These partial *GH* sequences were deposited in GenBank (accession numbers: *D. punctatus*: HM245614; *M. americana*: HM245611, *M. chrysops*: HM245615, *M. mississipiensis*: HM245612, and *M. saxatilis*: HM245613).

3.2 Repeat sequences in non coding regions

For all three sea bass genes, SSR loci were detected in their proximal promoter and/or their intronic sequences (Fig. 2). Only one SSR was detected in both *dlSL* and *dlIGF-1*, whereas two different SSRs ($(AC)_{23}$ in 5′ region and a composite $(GCAC)_{12}(AC)_4$ in intron 4) and one minisatellite ($((GTACTACACT)_{78}$ in intron 3) were identified along the *dlGH* sequence (Fig. 2). For the *dlSL* and *dlIGF-1* genes, those SSRs were subsequently named *μSL* and *μIGF-1*, respectively, and then assayed for polymorphisms (Fig. 2; see below). The presence of these repeat elements recorded at *dlGH* was checked for other Perciformes species and the zebrafish, a Cypriniform, whose sequences are available (Fig. 3). Because zebrafish is a model species, it was considered a relevant outgroup summarizing observations of sequences available outside the Perciformes (details given in the Discussion). Across the Perciformes, these sequence repeats were not consistently present and only two of them (*μGH* and m*GH*; Fig. 2) also occurred outside the Moronidae family (Fig. 1). For *Morone* spp., the SSR in intron 4 of *dlGH* was a pure motif (AC, ¤ in Fig. 3). Compared to the *GH* gene, scarcer genomic information was available for the *SL* and *IGF-1* genes (seven and five genomic sequences recorded in Perciformes respectively; details not shown). No SSR was found in *SL* genes for most species, except for one microsatellite observed

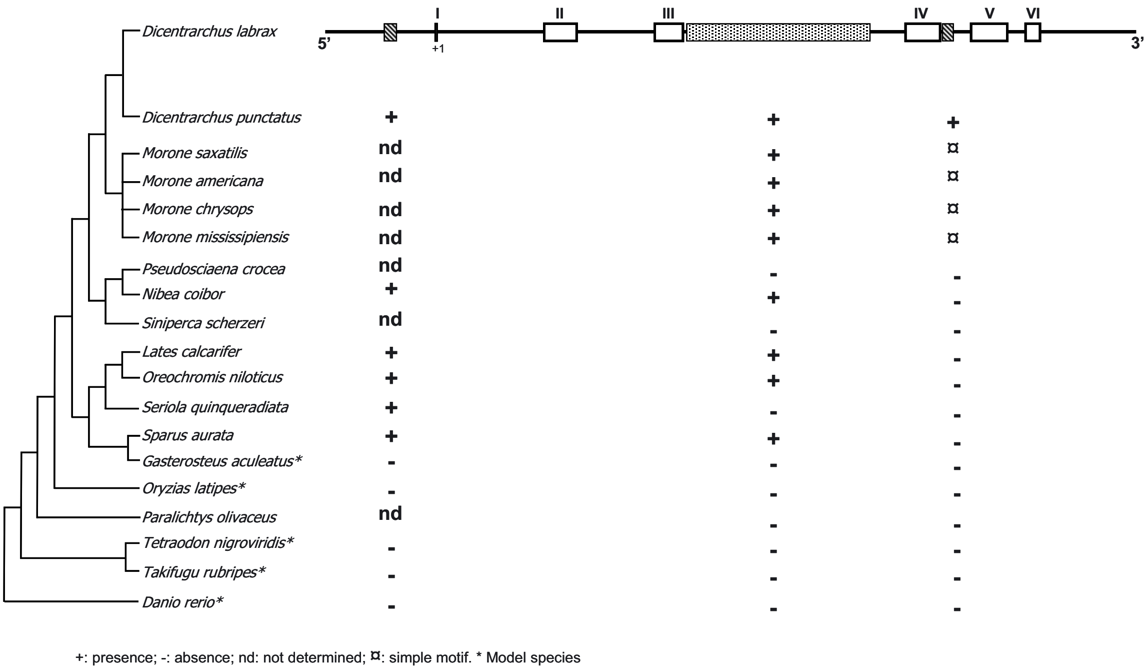

Fig. 3. Conservation of SSRs detected at dlGH for Perciformes. The phylogeny presented on the left of the figure is based on Smith and Craig's phylogeny of Perciformes (Smith and Craig 2007). Accession numbers for each species are: *Dicentrarchus labrax* (GQ918491); *Pseudosciaena crocea* (AY090592); *Nibea coibor* (FJ375311); *Siniperca scherzeri* (EF441623); *Lates calcarifer* (U16816); *Oreochromis niloticus* (= *Tilapia nilotica*: M84774); *Seriola quinqueradiata* (D50368); *Sparus aurata* (AF195646); *Gasterosteus aculeatus* (ENSGACG00000014829); *Oryzias latipes* (ENSORLG00000019556); *Paralichhtys olivaceus* (D29737); *Tetraodon nigroviridis* (ENSTNIG00000001811); *Takifugu rubripes* (ENSTRUG00000002048) and *(Brachy)danio rerio* (ENSDARG00000038185) is used as an outgroup. Data not shown for *Dicentrarchus punctatus, Morone saxatilis, M. americana, M. chrysops*; *M. mississipiensis.*

in the intron 2 of the *T. rubripes SL* gene (accession: ENSTRUG00000008210). The motif of this SSR was not homologous to that of *dlSL* (Fig. 2) but its location was identical according to the alignment of the flanking sequences (not shown). No conclusive homology was found for the SSR in intron 1 of the *IGF-1* gene between *D. labrax* and any other species. For this locus, degenerated motifs have been documented in *Gasterosteus aculeatus* (ENSGACG00000020042) and *Tetraodon nigroviridis* (ENSTNIG00000012663), but support for SSR conservation was low because of the degeneration and the low conservation of flanking sequences (details not shown). No sequence repeat was recorded for *Oryzias latipes*, *Takifugu rubripes*, and *Oreochromis niloticus* (ENSORLG00000016443, ENSTRUG00000014321, AF038123, respectively), making conservation of *dlIGF-1*-associated SSR too speculative to be graphically represented as for *dlGH*.

3.3 Genetic profiles of populations

Four loci were chosen for polymorphism analysis of the three genes: *µGH*, *mGH*, *µSL* and *µIGF-1* (Fig. 2). The *µGH* and *mGH* loci were both selected because (*i*) previous genetic studies in Perciformes used an homologous locus to *µGH* (Almuly et al. 2005, 2008; Zhang et al. 2009; Chaoui et al. 2009), and (*ii*) complex interactions were reported between this SSR and another intronic minisatellite in seabream, *Sparus aurata* (Almuly et al. 2000, 2008). Genetic profiles of the three wild and the two experimental (hatchery-derived) samples are shown in Table 1. Mean genetic diversity and mean allelic richness were both found to be larger in wild vs. cultivated samples (Table 1). No departure from HWE was detected for wild populations, whereas, as also expected, significant heterozygote deficiency occurred in cultivated samples (Table 1). No significant LD was detected at the single or multilocus level in wild populations, except between loci *µGH* and *mGH* ($p < 0.001$), which are physically very close (Fig. 2). For experimental samples, significant LD was demonstrated for the locus pairs *µGH-mGH*, *µSL-µIGF*, and *µIGF-µGH* (details not shown). Such LDs were not surprising in hatchery samples composed of related individuals.

3.4 Genetic differentiation among samples

Single and multilocus levels of genetic differentiation among pairs of wild populations are given in Table 2. No differentiation was observed between the Mediterranean populations (La Goulette vs. Sète), although genetic differentiation between each of the North Sea population and the Bay of Biscay population was detected ($F_{st} = 0.010$ and $F_{st} = 0.009$, $p < 0.05$). This differentiation only concerned locus *µSL* ($F_{st} = 0.019$, $p < 0.01$ and $F_{st} = 0.034$, $p < 0.001$). Significant genetic differentiation was found between Atlantic and Mediterranean populations, and between North Sea and Mediterranean populations, except at locus *µIGF-1* (Table 2).

Table 1. Summary of genetic diversity statistics. N: number of individuals; A: number of alleles; H: unbiased heterozygosity; F_{is}: index fixation; $\overline{H}$: mean unbiased heterozygosity; $\overline{A}$: mean number of allele.

	La Goulette	Sète	Bay of Biscay	Doel	Zeebrugge	Hatchery	
						Before acclimation	After acclimation
µGH							
N	24	38	29	27	27	24	25
A	13	12	13	13	11	7	6
H	0.873	0.844	0.830	0.797	0.780	0.786	0.764
Fis	0.144 *	–0.01 ns	0.002 ns	–0.022 ns	–0.046 ns	–0.281***	–0.317 ***
mGH							
N	28	38	29	26	29	18	29
A	10	11	9	9	8	3	4
H	0.687	0.643	0.467	0.609	0.599	0.637	0.748
Fis	–0.094 ns	–0.065 ns	0.041 ns	–0.141 ns	0.081 ns	–0.505 ***	–0.344 ***
µIGF1							
N	28	38	30	29	27	29	30
A	17	19	17	17	20	8	5
H	0.933	0.943	0.921	0.939	0.945	0.700	0.614
Fis	0.083 ns	–0.324 ns	0.024 ns	0.121 ***	0.060 ns	–0.345***	–0.198 **
µSL							
N	28	38	28	22	31	28	29
A	18	18	17	14	17	6	6
H	0.923	0.922	0.930	0.905	0.931	0.653	0.511
Fis	0.034 ns	0.002 ns	0.001 ns	0.098 ns	–0.005 ns	0.016 ns	–0.290 ***
Multilocus							
$\overline{A}$	14.50	15	13.75	13.25	14	6	5.25
$\overline{H}$	0.854	0.838	0.787	0.813	0.814	0.693	0.659
$\overline{Fis}$	–0.050 ns	–0.037 ns	0.014 ns	0.031 ns	0.020 ns	–0.277***	–0.292 ***

ns: non significant; *: $P < 0.05$; **: $P < 0.01$; ***: $p < 0.001$.

Table 2. Pairwise levels of genetic differentiation among wild populations: North Sea (Doel and Zeebrugge), Atlantic Ocean (Bay of Biscay), Western Mediterranean (Sète; La Goulette).

		La Goulette	Bay of Biscay	Zeebrugge	Doel
µIGF-1	Sète	0.001 ns	0.013 *	–0.002 ns	0.002 ns
	La Goulette		0.001 ns	0.002 ns	–0.004 ns
	Bay of Biscay			0.001 ns	–0.003 ns
	Zeebrugge				0.003 ns
µSL	Sète	–0.000 ns	0.019 **	0.026 **	0.014 *
	La Goulette		0.019 **	0.035 ***	0.024 *
	Bay of Biscay			0.019 **	0.034 ***
	Zeebrugge				0.016 *
µGH	Sète	–0.003 ns	0.070 ***	0.086 ***	0.086 ***
	La Goulette		0.073 ***	0.101 ***	0.099 ***
	Bay of Biscay			0.017 ns	–0.003 ns
	Zeebrugge				–0.001 ns
mGH	Sète	–0.006 ns	0.257 ***	0.209 ***	0.198 ***
	La Goulette		0.196 ***	0.150 ***	0.140 ***
	Bay of Biscay			–0.000 ns	0.002 ns
	Zeebrugge				–0.012 ns
Multilocus	Sète	–0.002 ns	0.081 ***	0.074 ***	0.70 ***
	La Goulette		0.065 ***	0.068 ***	0.061 ***
	Bay of Biscay			0.010 *	0.009 *
	Zeebrugge				0.003 ns

ns: non significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

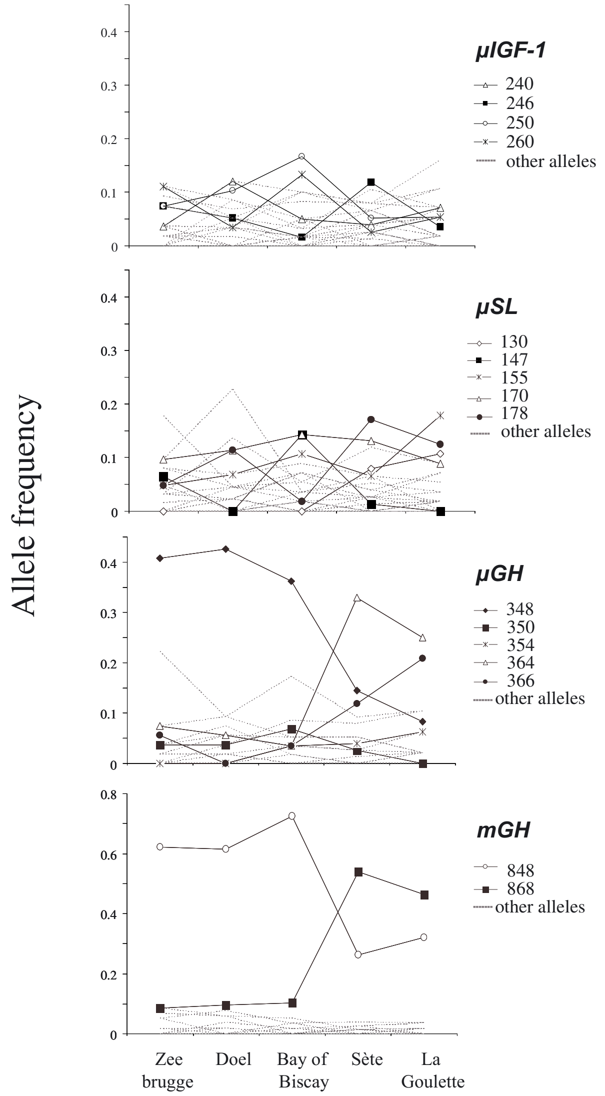

Fig. 4. Allelic frequency variation across populations at the four SSR loci (*µGH*, *mGH*, *µSL*, and *µIGF-1*). Alleles that most significantly contributed to F_{st} have been represented by coloured lines, other alleles by dashed grey lines. Sizes of alleles are given in base pairs. Each *y*-axis is provided with its own scale. See text and Table 2 for results pertaining to genetic differentiation among samples.

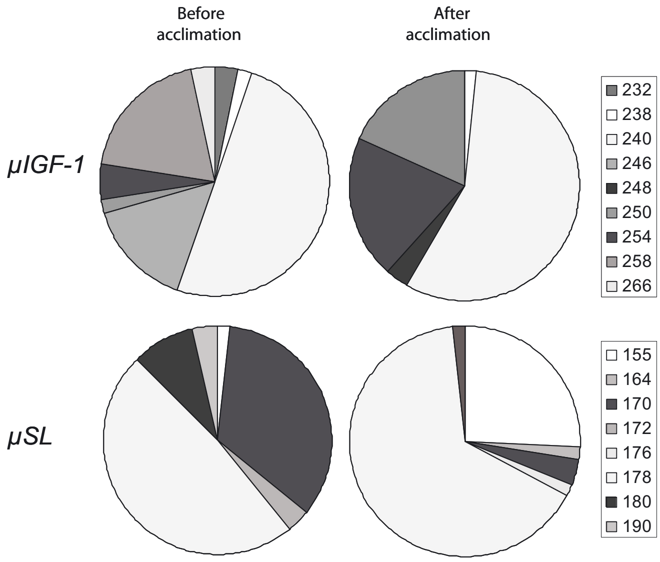

Fig. 5. Pie charts of allelic frequency distributions at the SSR loci that demonstrated significant allele frequency changes (*µSL* and *µIGF-1*) in cultured fishes before and after freshwater acclimation. Sizes of alleles are given for each locus (in base pairs). Data for *GH*-associated loci (i.e. no significant allele frequency changes) are not reported.

Genetic differentiation was especially driven by loci located in *dlGH* (*µGH* and *mGH*; Table 2). These two *GH*-associated markers revealed clear and broadly similar patterns of progressive longitudinal allelic frequency changes, which were either upward or downward depending on the alleles (Fig. 4).

Genetic differentiation among hatchery individuals also revealed overall multilocus genetic differentiation before and after salinity acclimation ($F_{st} = 0.0499$, $p < 0.001$). Differentiation was due to the SSRs associated with *dlIGF-1* and *dlSL* (*µIGF-1*: $F_{st} = 0.029$, $p < 0.05$; *µSL*: $F_{st} = 0.129$, $p < 0.001$) but not to loci associated with *dlGH* (*µGH*: $F_{st} = 0.018$; *mGH*: $F_{st} = 0.032$, $p > 0.05$ in both cases). A detailed analysis of allele frequency dynamics before and after the salinity challenge revealed that differentiation was due to a large increase in frequency of at least one rare allele, rather than to a change in frequency of the most frequent alleles (Fig. 5). At locus *µSL*, the shortest allele (155 bp) became about 15 times more frequent, with a concomitant decrease in allele frequencies of some larger alleles (e.g. 170 bp). At locus *µIGF-1*, the frequency of allele 254 was multiplied by a factor of almost 4 in individuals that survived acclimation as compared to individuals initially sampled in seawater (Fig. 5).

4 Discussion

Sea bass presents a puzzling situation in the field of population genomics. It is a target species of European marine aquaculture and studies on natural populations are numerous, including population genetics studies (reviewed in Chatain and Chavanne 2009). Many physiological studies have also been done on this species, including specific studies on the responses of sea bass to salinity (e.g. Jensen et al. 1998; Varsamos et al. 2002; Nebel et al. 2005; Giffard-Mena et al. 2008). As numerous genetic markers for population genomics studies have been developed in sea bass (reviewed in Volckaert et al. 2008), we might expect there to be a large bridge across disciplines, to provide more integrated biological studies of this species. This is definitely not the case yet, in contrast to other species of similar scientific and socio-economic interest (e.g., cod, *Gadus morhua*; Moen et al. 2008; Nielsen et al. 2009b; Rise et al. 2010). Lack of markers at physiologically significant candidate genes is certainly one explanation (see Introduction). In the present study, the exploitation of genomic scaffolds (Kuhl et al. 2010a) and traditional sequencing techniques allowed the development of such markers, and the investigation of their variation both in natural populations and among cultured individuals submitted to a salinity challenge that induced higher mortality rates in freshwater-challenged fishes.

4.1 Genomic sequences at physiologically important genes and associated markers

Full genomic sequences of three genes (*dlGH*, *dlSL*, and *dlIGF-1*) known to have a large physiological spectrum in fish were established for sea bass in this study. They complete previous sequences available for *dlGH* and *dlIGF-1* in which only mRNA – but not genomic – sequences were available (Doliana et al. 1992; Company et al. 2000, respectively), and *dlSL*, for which the first exon was missing (Terova et al. 2007). Associated hypervariable genetic markers (SSRs and minisatellite) located in non-coding regions close to or within these genes were identified (Fig. 2). Such gene-associated markers have proved to be efficient tools for genetic studies among Perciformes. The SSR homologous to μGH has been used in population studies of *S. aurata* in the Mediterranean (Chaoui et al. 2009), and in aquaculture studies on various Perciformes (Almuly et al. 2005, 2008; Zhang et al. 2009). To date in aquaculture studies, relationships of the alleles observed at *GH*-associated loci with growth rate (i.e. a genotype-phenotype relationship that may enhance performance and fitness; Fig. 1) have been suggested rather than firmly proved, possibly due to some antagonistic actions of genetic polymorphisms occurring at linked loci (Almuly et al. 2008). Concurrently, Li et al. (2009) reported a significant association between growth traits and the polymorphism of a locus located in the proximal promoter of the *IGF-1* gene in largemouth bass (*Micropterus salmoides*). We are not aware of any similar investigations dealing with markers associated with any *SL* gene, but the present release of the complete genomic sequences of these genes, together with description of conserved SSR markers, will undoubtedly provide opportunities to achieve such objectives in a broad range of species (Figs. 2 and 3). This is especially the case for other moronids, whose population management and aquaculture are of great concern in North America.

In this study, we concentrated mainly on the Perciforme group because homology among sequences located in the non-coding regions containing our markers is more easily maintained among closely related species (Fig. 3). Nevertheless, we also checked the literature for data indicating the presence of regulatory elements in non-coding regions of the *GH*, *IGF-1* and *SL* genes of other non-Perciformes. It is worth noting that a MAUI-like element has already been reported close to *GH* genes of fugu and salmonids (Poulter et al. 1999; Von Schalburg et al. 2008), and a minisatellite observed in the *GH* gene of Esocidae (Barnett et al. 2007). In this latter case, the minisatellite was located in the fourth intron rather than the third intron of sea bass and other species (Fig. 3). It presented a similar core structure to the microsatellite motif reported in Figure 2 (details not shown). Altogether, this indicates that such elements located in non-coding regions may have an active role in the regulation of gene expression outside the Perciformes. However, we did not find such evidence for other genes investigated in this study.

4.2 Natural populations

In this study, patterns of gene diversity at four gene-associated markers (μGH, *mGH*, μSL, and $\mu IGF\text{-}1$) turned out to be different from values previously reported at anonymous SSRs for wild populations (Naciri et al. 1999; Bahri-Sfar et al. 2000). Results revealed greater mean gene diversity and mean allelic richness in the two Mediterranean populations than in the Bay of Biscay population (Table 1). Although fewer populations are considered in this study, these patterns show opposite trends for these two genetic diversity statistics to those previously published for *D. labrax* (Naciri et al. 1999).

The significant genetic differentiation between Bay of Biscay + North Sea *vs.* Mediterranean populations previously reported in sea bass (SSRs: Naciri et al. 1999; mtDNA: Lemaire et al. 2005) was also found with our gene-associated markers (Table 2) but, as expected, not between Western Mediterranean populations belonging to the same hydrographic unit (Bahri-Sfar et al. 2000). The divide between the Atlantic (Bay of Biscay + North Sea) and the Western Mediterranean basins is thought to illustrate the incomplete re-homogenisation of these two genetic pools after secondary contact. Besides this similarity in patterns of genetic differentiation between the Atlantic-North Sea and Western Mediterranean populations, previous single locus F_{st} values ranged from 0.01 to 0.05 for anonymous SSRs, with mean multilocus $F_{st} \approx 0.02$ between the two basins (Naciri et al. 1999; see also Lemaire et al. 2005). Here, multilocus F_{st} as well as F_{st} values for *GH*-associated loci revealed genetic differentiation approximately three (μGH) and ten (*mGH*) times higher than previously found, whereas the μSL and $\mu IGF\text{-}1$ loci were in the range of F_{st} values for anonymous SSRs (Table 2). Such differentiation at a single gene might indicate selective effects (e.g. local adaptation) acting directly or indirectly on *GH*-associated loci. This hypothesis needs to be investigated further by looking at patterns of genetic differentiation over more loci, to test whether selection might have impacted genetic differentiation in sea bass at anonymous and gene-associated markers (e.g. Vasemägi et al. 2005). We could also evaluate the variability and directionality in patterns of introgression among these marker classes in this contact zone.

Concomitantly, results indicated low but significant genetic differentiation between the Bay of Biscay and the two North Sea populations at locus μSL (Table 2). Differentiation at SSR loci among north-eastern Atlantic populations has been never documented for sea bass (e.g. Bonhomme et al. 2002) except by Fritsch et al. (2007), who reported possible differentiation around the British Isles. However, the size of some samples was relatively low in this previous study ($n < 15$), possibly suggesting biases in estimates of genetic differentiation. Because of larger sample sizes and higher levels of genetic differentiation, such bias probably did not occur in the present study. Nevertheless, the cause of such differentiation (i.e. demography vs. selection) cannot be inferred from our data.

4.3 Genetic differentiation associated with the salinity challenge

Samples of farmed fish typically exhibit particular features linked with aquaculture practices. Thus, observed genetic diversity and allelic richness were lower in those samples (Table 1). As also expected, significant LD was found in the samples that were composed of related individuals. LD might possibly have been exacerbated if the salinity challenge induced differential and selective mortality among specific allelic combinations. In this study, the mortality rate was found to be greater in fish exposed to fresh water rather than seawater (53% vs. 22%, respectively) and the significant genetic differentiation observed at the *μSL* and *μIGF-1* loci supports the idea of such selective mortality, with some alleles at these either selected or counter-selected during our four month acclimation process (Fig. 5). As an example, the shortest *μSL* allele (155 bp) became about 15 times more frequent after acclimation, possibly reflecting selection; some larger alleles being counter-selected. Streelman and Kocher (2002) reported a similar observation, favouring shortest alleles of the prolactin gene in freshwater-acclimated Mozambique tilapias. Prolactin is involved in freshwater acclimation and has close phylogenetic proximity with SL (Huising et al. 2006).

Genetic differentiation amongst cultured fish before and after a salinity challenge has been investigated previously in sea bass (Allegrucci et al. 1994, 1995). It has also been demonstrated that most genetic markers of such differentiation can also be identified in wild populations, and that there is a contrast between individuals captured in marine and lagoon environments (Allegrucci et al. 1997; Lemaire et al. 2000). However, to date genetic differentiation amongst individuals submitted to salinity challenges have been identified either with allozymes (i.e. proteins; Allegrucci et al. 1994) or RAPDs (Allegrucci et al. 1995). Thus, the genetic differentiation at *μSL* and *μIGF-1* loci represents the first compelling evidence of acclimation-based differentiation at non-anonymous DNA markers. It might seem surprising that these loci are only indirectly involved in fish osmoregulation (e.g. Mancera and McCormick 1998b; Vargas-Chacoff et al. 2009), whereas pituitary GH exerts direct osmoregulatory actions (e.g. Sakamoto and Hirano 1993; Mancera and McCormick 2007). For *dlSL*, it has also been reported that changes in gene expression can be induced by confinement in cultured fish (Laiz-Carrión et al. 2009; Uchida et al. 2009). Thus, it is possible that differentiation at this gene was not only driven by salinity, but also by fish density. Finally, as hatchery fishes are derived from families, we need to be cautious in interpreting levels of genetic differentiation at these two loci. Particular families, as opposed to particular alleles, might have been selected during the acclimation process, resulting in inflated genetic differentiation. The way in which any such "family effect" might influence results needs to be investigated further, including extending study to more loci.

5 Conclusion

New markers are now available in sea bass, and probably other Perciforms, for candidate gene loci associated with osmoregulation and other adaptive physiological functions. These markers show promise for studies of the genotype-phenotype relationship in both cultured populations and wild fishes (e.g. exploration of relationships between observed allelic distributions and physiological performance of individuals as measured by gene expression; Fig. 1). The availability of a draft genome (Kuhl et al. 2010a) should also provide new opportunities for population genomics studies in sea bass.

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Supplementary materials

Annexes 1 and 2 are available after the references.

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Supplementary materials

Annexe. 1. Full list of primers used to amplify successive gene sequences along genes investigated in this study. PCR conditions are reported in the main text.

Gene	Primer name	Sequence	Tm
GH	DLGH_5′For	ggatatgtggctcaatagag	58
	DLGH_5′Fbis	gctacagcactgtcagatag	60
	DLGH_5′Rev	cactgataatggacagttggac	64
	DLGH_5′Revbis	gccatttcacagataatgact	58
	DLGH_5′msF	aaacagaactggctgcagtg	60
	DLGH_5′msR	atcaggttcaggttcaggttc	62
	DLGH_int1For	gtgatcagtcgggttcaggt	62
	DLGH_ex2Rev	ctgtgattggctgagaagac	60
	DLGH_ex2For	gtcttctcagccaatcacag	60
	DLGH_int2-3For	tgcgtttgttaggagagctc	60
	DLGH_ex3F	tcatcagccccatcgacaagc	60
	DLGH_ex4R	gataggagatagacagcagc	58
	DLGH_ex3Rev	ttgttcagttgtcgctgctc	60
	DLGH_ex3Revbis	tcacagaactgcgttgtgtc	60
	DLGH_ex4F	tctatcgatcggttgcttcc	60
	DLGH_3′R	atccgaatgcaacacagcac	60
IGF-1	DLIGF1_5′F	tttgtcttgcggagacccg	60
	DLIGF1_ex2R	acgcacagcagtagtgagag	62
	DLIGF1_int1F	aacactgctgctcaatggcca	64
SL	DLSL_ex2For	taccgcgtgtctgaagaatc	60
	DLSL_int2Rev	ttactccactgtaggcctgc	62
	DLSL_ex3For	tcaccaaagccttacccatc	60
	DLSL_ex4Rev	ttgttgagcagcatttcagg	58
	DLSL_ex4For	tgctggttcagtcgtggatc	62
	DLSL_ex5Rev	ccagtatgatgtgccaccag	62
	DLSL_int2msF	ggttaaggataaataggtcag	58
	DLSL_int2msR	acaatccatacaagcatggg	58

Annexe. 2. Primers and PCR conditions for tandem repeats analysed in *dlGH*, *dlSL* and *dlIGF-1*.

Locus	Motif	5′-3′ Primer		PCR mix: Buffer	MgCl$_2$ (mM)	dNTP (mM)	Each primer (mM)	Taq(U)	Final volume (µl)	Amplification: denaturation	30 cycles			Final elongation
µGH	(AC)$_{23}$	DLGH5′msFm	AAACAGAACTGGCTGCAGTG	5X	15	0.8	1	0.2	10	94°	94°	60°	72°	72°
		DLGH5′msR	ATCAGGTTCAGGTTCAGGTTC							2 mm	10 s	10 s	20 s	5 mm
mGH	(GTACTACACT)$_{78}$	DLGHex3Fm	TCTTCCTGCAGGATTTCTGC	5X	37.5	2	2.5	0.5	25	94°	94°	58°	72°	72°
		DLGHex4R	GTAAAATCTGGTTCCTGGC							2 mm	30 s	30 s	1 mm	5 mm
µSL	(TG)$_{30}$	DLSLint2msFm	GGTTAAGGATAAATAGGTCAG	5X	37.5	2	5	0.5	25	94°	94°	58°	72°	72°
		DLSLint2msR	TTACTCCACTGTAGGCCTGC							2 mm	30 s	30 s	1 mm	5 mm
µIGF-1	(CT)$_{28}$CC(CT)$_5$	DLIGF1int1msFm	AACACTGCTGCTCAATGGCCA	5X	37.5	2	5	0.5	25	94°	94°	58°	72°	72°
										2 mm	30 s	30 s	1 mm	5 mm