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Genetic Variation of Insulin-like Growth Factor II (*IGF-II*) Gene and its Associations with Growth Traits in European Sea bass (*Dicentrarchus labrax*)

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Abstract

Insulin-like growth factor II (IGF-II) is a peptide hormone which plays a crucial role in the growth and metabolic regulations of vertebrates. In this study, we identified single nucleotide polymorphisms on *IGF-II* gene in the European sea bass (*Dicentrarchus labrax*) and revealed the potential association between the genotypes of *IGF-II* gene and growth traits in 150 European sea bass. The polymorphisms were determined using DNA sequencing and PCR-RFLP methods in two different regions of *IGF-II* gene. An AT indel and two SNPs as g.5127711C>G and g.5127731G>T in the intron 2 region, and one SNP as g.5129188C>T in the intron 3 region of *IGF-II* gene in the European sea bass were reported for the first time with this study. Two different genotypes (GG, TG) were observed and the G allele was predominant (65%) in the *IGF-II-Nde*I locus. Associations between the genotypes of *IGF-II-Nde*I locus and body weight and total length were statistically significant (P<0.05). Body weight and total length in each group were higher for GG genotype of *IGF-II-Nde*I locus (p<0.05). In conclusion, the *IGF-II-Nde*I locus had a significant effect on the body weight and total length which could be useful for sea bass selection and breeding for marker-assisted selection program.

Introduction

The European sea bass (*Dicentrarchus labrax*), is a marine teleost fish, distributed along the European coastal area of Atlantic Ocean and Mediterranean Sea. Its intensive exploitation as an aquaculture species has become very common during the last decade and the production is concentrated predominantly in the Mediterranean basin, as gilthead sea bream (*Sparus aurata*) (Vandeputte, Quillet, & Chatain, 2012). The European sea bass production has increased 24% and reached up to 191 thousand tons in the last 5 years. The commercial value of sea bass aquaculture production was approximately 1.1 billion dollars in 2016 (FAO, 2018). European sea bass reaches the market size in 16-24 months under the Mediterranean environmental conditions (Rad & Şen, 2016). Thus, growth rate is the most important trait in the economical aspect of the sea bass production. Havenstein, Ferket, & Qureshi (2003) reported that farm animal production could be increased by 600% in the systems integrated with the breeding and genetic technologies. In the last few decades, molecular markers have been utilized to improve economic traits that were required for a fast and effective production (Schlicht *et al.,* 2019). Therefore, using molecular markers in relation to growth traits is an essential step for the success of selective breeding programs for the European sea bass production (Louro *et al.,* 2016).

Molecular markers aimed to detect genetic variation in terms of candidate genes of known or inferred functions that related to the trait of interest were called quantitative trait locus (QTL) markers (Lynch & Walsh, 1997; Tao & Boulding, 2003; Desantis & Jerry, 2007). QTL loci in the genome controlling specific traits, such as growth-axis, were investigated employing different molecular markers (Juhua *et al.,* 2010). Single nucleotide polymorphisms (SNPs) were widely studied markers to detect the genetic variation in the genome (Vykoukalova, Knoll, Dvorak, & Cepica, 2006; Berkowicz *et al.,* 2011; Huang *et al.,* 2014).

One of the most predominantly analyzed genes related with growth was *IGF-II* which had a known regulatory mutation that caused a major QTL effect on 30 % muscle growth in pig (Van Laere *et al.,* 2003; Louro *et al.,* 2019). *IGF-II* encoded a peptide hormone with insulin like growth factor, which played an important role in the growth of vertebrates and the regulation of their metabolism (Cornish *et al.,* 2007; Harris & Westwood, 2012). Various studies revealed associations between *IGF-II* gene polymorphism and economic traits of pigs, cattle, buffalo and chicken (Li, Wang, Zhao, & Wang, 2004; Abo-Al-Ela, Abu El-Magd, Abeer, El-Nahas, & Mansour, 2015; Yan *et al.,* 2015). Radaelli, Patruno, Maccatrozzo, & Funkenstein (2003) revealed that *IGF-II* was expressed not only in early embryonic development but also during adult life in fish. In another research, *IGF-II* gene had both metabolic and myogenic effects in muscle cells of rainbow trout (*Oncorhynchus mykiss*) (Codina *et al.,* 2008). Terova *et al.* (2007) found that mitogens of the regions related to *IGFI* and *IGF-II* had important role in the growth and development of European sea bass. *IGF-II* was also known to be an imprinted gene with paternal inherited allele expression in both eutherians and marsupial bi-allelic expression was reported in two live-bearing fishes (Lawton *et al.,* 2005; Louro *et al.,* 2018). Peterson, Small, Waldbieser, & Bosworth (2008) revealed that the *IGF-II* mRNA levels were greater in muscle and liver of fast-growing fish compared to slow-growing fish in channel catfish (*Ictalurus punctatus*). On the contrary, *IGF-II* expression levels in liver found lower in fast-growing European sea bass compared to slow-growing fish (Louro *et al.,* 2018). In another study, two markers in the *IGF-II* gene region of a genetically improved farmed male tilapia (*Oreochromis niloticus*) population were found to be closely related with growth (Juhua *et al.,* 2010). Li *et al.* (2012) reported significant associations between genotypes and growth traits of largemouth bass (*Micropterus salmoides*). These findings indicate that *IGF-II* gene is a good candidate gene for MAS programs to improve fish growth traits.

The aims of the presented study were: (i) to assess polymorphism in the *IGF-II* gene of the European sea bass (*Dicentrarchus labrax*), using DNA sequencing and PCR-RFLP methods, and (ii) to test the association between the possible polymorphisms and some growth characteristics that would be used in selective breeding programs.

Materials and Methods

Sampling and DNA Isolation

Fish that were hatched at the same time and reared under the same environmental conditions (natural photoperiod in off-shore cages, 15kg/m3 harvest size density) were obtained when they were 24 months old from a fish farm located in Karaburun Bay-İzmir by a private Fish Processing Factory in İzmir in order to be processed. We sampled 150 individuals from the processing company. Feeding was done with the compound diet commercially produced for sea bass. Fish had been sized until they were 2g. The body weight (W) and total length (TL) of each sample were measured as a first step and commercial grading (4 groups of different weights) was performed. Sampling of fish was done randomly from the male individuals due to excess of males in culture as a consequence of the environments used in culture, interacting with a complex system where both environmental and genetic factors govern sex determination in sea bass (Vandeputte *et al.,* 2012). Then, muscle tissue samples were taken and stored at -20°C until DNA extraction. Genomic DNA was extracted by using a commercial DNA isolation kit (EURX Gene Matrix Tissue- Bacterial DNA Purification Kit, Poland) according to manufacturer's instructions. The quality and quantity of DNA samples were checked by 1% agarose gel electrophoresis and evaluated visually and by UV spectrophotometry (MaestroGenNano).

PCR Amplification of *IGF-II* Gene

The exon and intron 2 regions (900 bp) of *IGF-II* gene (*IGF-II*-1) and exon and intron 3 regions (374 bp) of *IGF-II* gene (*IGF-II*-2) were amplified by PCR (Table 1). The primer sequences of these two regions were designed based on the *Dicentrarchus labrax* DNA sequence retrieved from GenBank (Accession number HG916846) (Contig CBXY010013989.1) using Primer-BLAST algorithm (https:// www.ncbi.nlm.nih.gov/tools/primer-blast/), and given in Table 1. For amplification reactions, the 35 µL PCR volume contained: 50 ng genomic DNA, 0.5 µM of each primer, 1× PCR Buffer ($(NH_4)_2SO_4$), 200 µM dNTP, 2.5 mM $MgCl_2$ and 1 U of Taq DNA polymerase (Taq DNA Polymerase, Thermo Scientific, US). The cycling protocol was 5 min at 94°C for initial denaturation, 35 cycles of amplification; 94°C for 45 s, 58- 60°C annealing for 60 s, 72°C for 60s and 10 min at 72°C for final extension. Afterwards, the PCR products were checked on 2 % agarose gel using horizontal electrophoresis and the gels were stained using SafeView™ Classic (Applied Biological Material Inc. Canada).

Table 1. Primers used to amplify European sea bass *IGF-II* gene

Primer Name	Primer Sequence	Annealing Temperature (^{0}C)	Product Size	Amplified Region
IGF-II-1	F: 5'-ATGTCTTCGTCCAGTCGTG-3' R: 5'-AAAGCAGTGGGTGGGACATAG-3'	58	900 bp	Exon 2 and Intron 2
IGF-II-2	F: 5'-ACAACAGACGGACCCAGAAC-3' R: 5'-CGACAGTCAGCACTTTGCAT-3'	60	374 bp	Exon 3 and Intron 3

Genotyping of SNPs by Restriction Fragment Length Polymorphism (RFLP)

The selection of the restriction enzymes for RFLP analysis was performed via NEBcutterV2.0 (http://nc2.neb.com/NEBcutter2/) using studied *IGF-II* gene regions. The PCR products of the *IGF-II*-1 and *IGF-II*-2 gene regions were digested by *Nde*I (Thermo Scientific, Cat # FD0584, Lot # 00430875) (CA'TATG) and *Mbo*II (Thermo Scientific, Cat # FD0824, Lot # 00439340) (GAAGA(N)8') restriction enzymes, respectively according to the protocols recommended by the manufacturer. After digestion, the products were visually checked by agarose gel electrophoresis using 3% agarose (Prona, EU) gel stained with SafeView™ Classic (Applied Biological Material Inc. Canada).

Sequence Analysis

Randomly chosen 15 samples of PCR products (total of 30 samples) from each considered region were sent to a private laboratory for DNA sequencing analysis in order to investigate additional variation of the *IGF-II* gene regions. PCR products were sequenced by automated capillary electrophoresis system (Applied Biosystems, 3500xL Genetic Analyzer, Thermo Fisher Scientific, UK). The electropherogram were carefully checked by using ChromasPro Version 2.1.8 (Technelysium Pty. Ltd. Australia). Afterwards, the sequences were formed for each sample individually by aligning its forward and reverse sequences and a final data file consisting from consensus sequences for each sample was obtained. Moreover, the trimmed sequence file of *IGF-II* fragments was analyzed by the MEGA6 software (Molecular Evolutionary Genetics Analysis, version 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

Statistical Analysis

Hardy-Weinberg equilibrium of the population was tested via POPGENE (v.1.32) (Yeh, Yang, & Boyle, 2000). The association between the genotypes (*IGF-II-Nde*I locus) and economically important traits such as body weight (W) and total length (TL) in European sea bass was determined (via SPSS Inc. V. 18.0, IBM, Chicago, IL, 2009) according to the General Linear Model and an alpha value of 0.05 was chosen as significance level.

Linear Model I = $Y_{ij} = \mu + B_i + G_j + e_{ij}$

where Y_{ij} represents the body weight (W) and total length (TL); μ represents the average; B_i represents group effect, G_j represents the effect of *IGF-II* genotype and e_{ij} is the random error.

Results

In *IGF-II*-1 gene region, an AT insertion/deletion was observed in the 389-390th (5127733-5127734th/AT) positions (Figure 1) and two more SNP positions were determined based on the sequencing results. G$\rightarrow$T transversion was observed on the 387th position (g.5127731G>T) and C$\rightarrow$G transversion was observed on the 367th position (g.5127711C>G) in the intron 2 region of *IGF-II*-1 fragment (Figure 2). Likewise, the sequencing of *IGF-II*-2 gene region has revealed a C$\rightarrow$T transition on the 169th position (g.5129188 C>T) of the intron 3 of *IGF-II* gene (Figure 3).

Two genotypes were identified in the *IGF-II* gene with the *Nde*I restriction enzyme: GG homozygotes (508, 392 bp), TG heterozygotes (900; 508, 392 bp) (Figure 4). According to the *Nde*I restriction of the *IGF-II*-1 fragment, among all the samples, 44 individuals had GG genotype and 106 individuals had TG genotype while TT genotype was not observed. The allele frequencies were estimated as 0.35 for T allele and 0.65 for G allele. We have estimated the expected genotype frequencies and tested for the deviation from Hardy-Weinberg equilibrium using Chi-square test, which revealed a significant deviation ($P<0.01$). These results were given in the Table 2. The RFLP analyze result of *IGF-II*-2 gene region using *Mbo*II restriction enzyme gave only one haplotype and did not reveal any polymorphism.

The Association between *IGF-II*/*Nde*I Genotypes and Growth Traits

Phenotypic characteristics of the European sea bass individuals were recorded when they were 24 months old. The relationships between the genotypes of *IGF-II-Nde*I locus and body weight and total length scores were summarized in Table 3. For *IGF-II-Nde*I locus, a result of the association analysis based on General Linear Model, the observed difference between the groups was significant ($P<0.05$). The individuals that have GG genotypes, had higher body weight values in

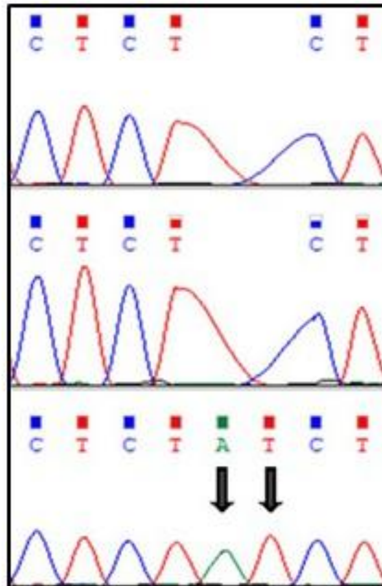

Figure 1. Partial sequence of *IGF-II* gene showing AT insertion\deletion in 2ndintron region.

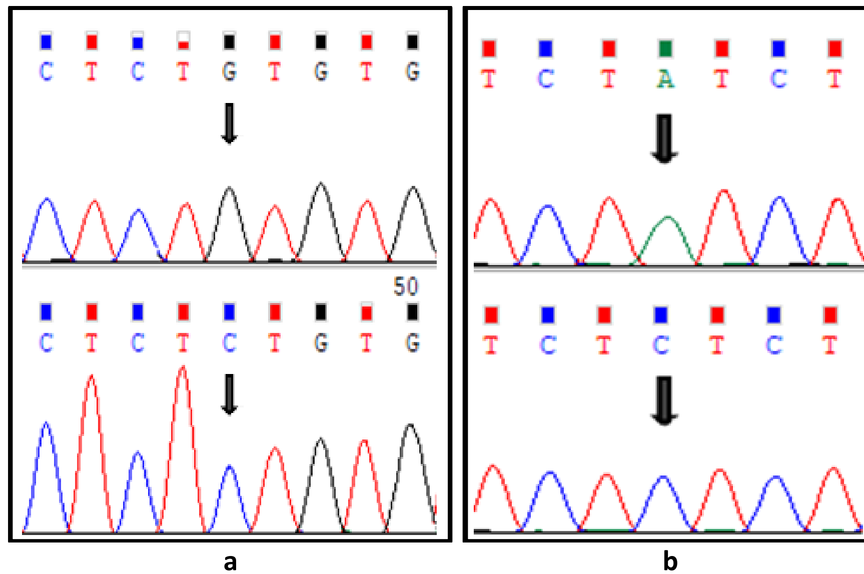

Figure 2. Partial sequence of *IGF-II* gene showing a) g.5127711C>G (g.367C>G), b) g.5127731G>T (g.387C>A) (antisense sequence) polymorphisms in 2nd intron region.

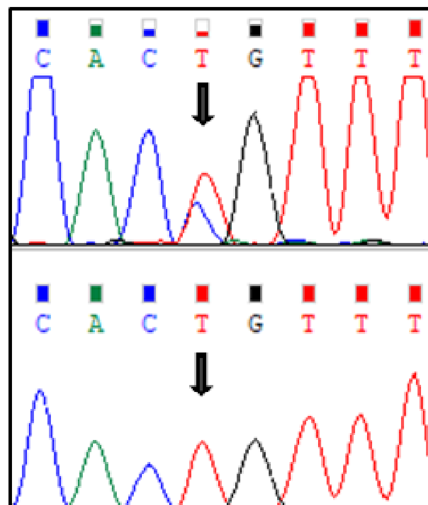

Figure 3. Partial sequence of *IGF-II* gene showing g.5129188 C>T, polymorphism in 3rdintron region.

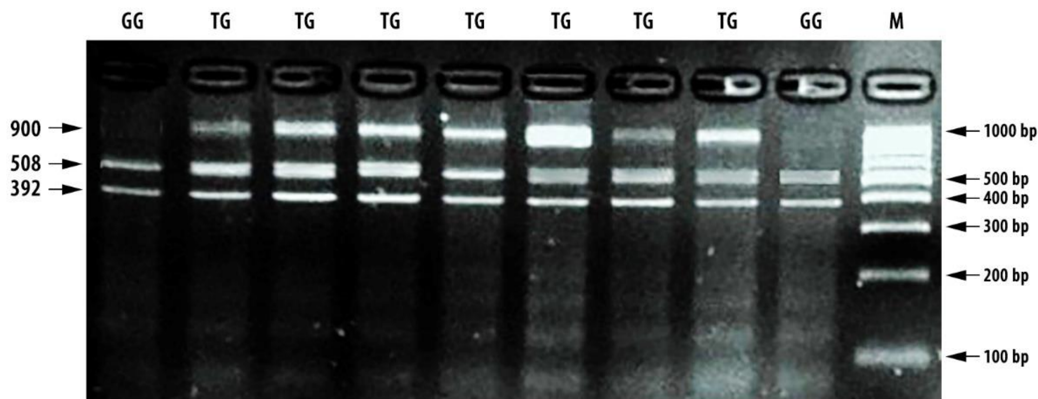

Figure 4. Genotypes of *IGF-II*-1 fragment of *Dicentrarchus labrax*, M; Marker, *Nde*I digestion of the *IGF-II*-1 fragment, lines 1 and 9 homozygote: GG (508, 392 bp); lane 2-8: TG heterozygote (900; 508, 392 bp).

Table 2. The observed genotypes and allele frequencies obtained from RFLP analysis of *IGF-II*-2 fragment using *Nde*I restriction enzyme. The estimated expected genotype frequencies and Chi-square test result are also given

Locus		*IGF-II- NdeI* Genotypes			Allele Frequency		χ2
		GG	TG	TT	T	G	
IGF-II-NdeI	Obs.	44	106	0	0.35	0.65	45.1
	Exp.	63.4	68.3	18.4			(p<0.001)

Table 3. The association of *Nde*I polymorphisms of *IGF-II* gene with the body weight and total length in European sea bass

Trait	Body Weight (W) (g)		Total Length (TL) (cm)	
Genotype	GG	TG	GG	TG
1.Group	305.512±12.076[a]	296.755±11.095[b]	29.763±1.304 [a]	29.446±0.632 [b]
2. Group	367.304± 9.326[a]	358.547±9.178[b]	32.571±1.350 [a]	31.894±0.842 [b]
3.Group	515.792±10.343 [a]	507.035±8.315[b]	37.750±0.954 [a]	34.870±0.700 [b]
4.Group	687.425±16.880[a]	678.669±14.255[b]	43.717±1.458 [a]	38.406±0.739 [b]
P-value	0.000		0.04	

Note. Values in the same row with different superscripts are significantly different (P<0.05).

each group (P<0.05). In addition, the total lengths of fish with GG genotypes for the *IGF-II-Nde*I were significantly higher than the TG genotype in each group (P<0.05) at the harvest time. These findings suggested that for fish with *IGF-II-Nde*I locus had a significant effect on body weight and total length which could be useful for sea bass selective breeding programs considering marker-assisted selection (MAS).

Discussion

In this study, we investigated 2nd, 3rd exon and 2nd, 3rd intron of the *IGF-II* gene and its polymorphism in the European sea bass. An AT insertion\deletion was found in intron 2 region of *IFG-II*-1 gene. Juhua *et al.,* (2010) also found an insertion in the middle of two intron regions of *IGF-II* gene of Nile tilapia. The intron regions which have many repetitive sequences and a higher AT content may not be suitable for DNA sequencing by Sanger method. However, NGS (Next Generation Sequencing) technologies can help to improve high-throughput SNP analysis. Besides Taq DNA polymerase and PCR chemicals should be developed specifically for AT/GC rich regions which could help to perform more precise PCR amplifications and sequence analysis.

The two SNPs as g.5127711C>G and g.5127731G>T were determined in the intron 2 region and one SNP as g.5129188T>C was observed in the intron 3 region of *IGF-II* gene. SNPs in intronic regions may regulate gene expression, especially when they are located close to exon regions (De-Santis & Jerry, 2007; Tsai, Hamilton, Guy, & Houston, 2014). The ends of intron regions have an important role in RNA splicing (Fedorova & Fedorov, 2003). Tao and Boulding (2003) revealed that the presence of a G nucleotide in the SNP allele at the intron conjunction could effect the growth traits in Arctic charr

(*Salvelinus alpinus*). This SNP could cause the differential splicing of mRNA and greater expression of PACAP in fish possessing it. Several studies have shown that SNPs in the intron regions of the growth related genes, had influence on fish growth characteristics e.g. in the farmed Atlantic salmon (*Salmo salar*) (Tsai *et al.,* 2014) and rainbow trout (Panicz, Zych, & Grzesiak, 2014). Researches have carried out studies to investigate the associations between *IGF-II* gene variants with back fat, body weight, fat composition, and other quantitative characteristics in chicken, beef and pigs (Van Laere *et al.,* 2003; Li *et al.,* 2004; Vykoukalova *et al.,* 2006; Zhang, Chen, Li, Lei, & Xue, 2007). Van Laere *et al.* (2003) proved a causal relationship between a SNP in intron 3 region of *IGF-II* gene and a QTL effect in pigs. Intron regions are more polymorphic than exon regions (Tsai *et al.,* 2014). However, only a few researches investigated the relationships between *IGF-II* gene region and growth characteristics in fish. Juhua *et al.* (2010) determined the association between the *IGF-II* gene and growth trait in GIFT strain of tilapia and they identified nine polymorphisms in the 1st, 3rd intron and four silent mutations in the 2nd, 3rd exon regions. Li *et al.* (2012) reported four SNPs in the *IGF-II* gene of largemouth bass. They have not found significant association between genotypes of *IGF-II* gene and growth traits in largemouth bass but on the other hand they found relationship with two diplotypes and growth traits in *IGF-II* gene region ($p<0.05$). Khatab *et al.* (2014), determined 14 SNPs in *IGF-II* gene region of large size Nile tilapia but they did not conduct an association study.

Louro *et al.* (2018) revealed a candidate function of *IGF-II* in growth regulation of European sea bass. In another study, Louro *et al.* (2016) found that the sea bass LG6 (linkage group 6) has a high quantitative effect on the growth axis such as *IGF-II* and leptin. Therefore, in our study we have chosen to identify *IGF-II* gene located on LG6 (HG916846). We used two different restriction enzymes for genotyping with PCR-RFLP and only one of them was found non-polymorphic. Two different genotypes (GG, TG) were observed and the G allele was predominant for *IGF-II-Nde*I locus. We think that the absence of TT genotype may be due to the elimination (on sizing) of slow-growing individuals from the population by human during aquaculture process. We found that the *IGF-II-Nde*I locus had a significant effect on body weight and total length which could be useful for carrying out marker assisted selection (MAS) in a sea bass selective breeding program.

Molecular markers have been used more commonly in the breeding activities to shorten the long and labor-intensive periods of the species taken into production in aquaculture and also to increase the efficiency of the selective breeding program. There are many environmental variance components in aquaculture, due to intensive and time-consuming production process and thus, MAS has a significant potential for identifying individuals with genetically superior growth characteristics (Tao & Boulding, 2003). Many researches have been carried out to reveal the association between gene polymorphism with economic traits in aquaculture. As a conclusion of this study *IGF-II* gene is a strong candidate gene for the growth traits in the European sea bass and this result is also supported by the previous studies of other fish species and farm animals. We strongly recommend using this region as a marker in breeding programs planned for sea bass.

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