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Finding 16S rRNA gene-based SNPs for the genetic traceability of commercial species belonging to Gadiformes

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ABSTRACT: A SNPs (Single Nucleotide Polymorphism) based analysis was developed to differentiate four economically important species belonging to the Gadiformes order: Pacific cod *Gadus macrocephalus,* Atlantic cod *Gadus morhua,* Haddock *Melanogrammus aeglefinus* and Ling *Molva molva.* A 430bp fragment of the 16s rRNA gene was amplified using interspecific conserved primer and sequenced. The sequences were aligned and analyzed for the presence of SNPs; three SNPs (MerSNP1, MerSNP7 and MerSNP9) were identified and selected to allow discrimination between the four species. Aplotypes were TCC, CCC, CAT and CAC for Pacific cod, Atlantic cod, Haddock and Ling respectively. Confirmation of results was achieved by sequencing 16s rRNA gene fragments of 16 *G. morhua*, 7 *G. macrocephalus*, 15 *M. aeglefinus* and 5 *M. molva* samples collected at different fish catching campaign. Nucleotide sequence of 16s rRNA mitochondial gene has been shown to be a useful tool to allow rapid reliable and fully automatable for discrimination of 4 economically important species in fisheries industry.

Key words: Genetic traceability, Gadiformes, SNPs, 16S rRNA.

INTRODUCTION- The identification of fish species in processed seafood has become a very important issue nowadays. EU Regulation 2065/2001 states that fishery and aquaculture products shall be labeled with species name in the package. While morphological identification is generally easy for fresh and unprocessed fishes, for many fish species there is the possibility of fraudolent or accidental species substitution when products are imported or sold without informative morphological characters (i.e. fresh or frozen portions of fish, precooked fillets, etc.). Many molecular methodologies have been developed for different fish species identification, isoelectrofocusing (IEF), two-dimensional electrophoresis (2DE) and SDS-PAGE have been previously used for identification of some hake species (Mackie, 1990; Sotelo *et al.,* 1993) but the low allelic variation of allozymes as well as the low reliability in processed foods do not always permit the routinary use of this techniques in species identification. DNA-based analysis have recently been widely used in fish species discrimination for their ability to discriminate between closely related species, belonging to the same family or genus (Lockley, and Bradsley, 2000,), and for their reproducibility. Mitochondrial genes such as ITS-1 rDNA, cytb and 16s rRNA genes have been already proved to be good markers for species identification in fishes (Quintero *et al.*, 1998, Perez *et al.*, 2005, Aranishi *et al.*, 2005) as well as for phylogenetic analysis and population genetics. In this study we developed a SNPs based analysis in a highly conserved 16s rRNA gene region of 4 different species belonging to the Gadiformes order (Pacific cod *Gadus macrocephalus,* Atlantic cod *Gadus morhua,* Haddock *Melanogrammus aeglefinus* and Ling *Molva molva*) to discriminate with a single analysis the four different species. Direct sequencing of amplicons amplified with interspecific conserved primers is a completely automatable technique for high-throughput routine surveys.

MATERIAL AND METHODS - Samples were kindly provided by Rivamar (Taglio di Po – RO, Italy) collected by fishery catch in Alaska (*G. macrocephalus*) and Iceland (*G. morhua, M. aeglefinus* and *M. molva*) and imported as frozen fillets (*M. aeglefinus* and *G. macrocephalus*) salted fillets (*M. molva*) or dry (*G. morhua*). Genomic and mitochondrial DNA was purified using the DNeasy Tissue Kit (Qiagen) with minor modifications, 20 mg of frozen tissue was used as starting material. DNA was resuspended in TE buffer (10mM Tris-Cl, 0,5 mM EDTA). PCR

amplification of the partial 16s rRNA gene was carried out in 50ml of Red Taq Buffer 1X (Sigma) containing 20ng of template DNA, 200mM of each dNTPs, 1 unit RedTaq DNA polymerase (Sigma), 2,1 mM $MgCl_2$ and 0,2 mM of each primer. 16s rRNA sequences of different Gadiformes fishes available at Genbank (accession numbers: AJ315613, AJ517841, AJ315620, AJ315630, AJ517843) were aligned to design interspecific conserved primers. Intersepecific conserved primers were: MER 16s fwd (5'-AAGAGCCCTTTA-GTTTGTAACC-3') and MER 16s rev (5'-GCATAAGACAGCCTGGTAGAG-3'). PCR reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems) with an initial denaturing step of 5 min at 94°C, followed by 30 cycles with denaturation at 94°C for 20 s, annealing at 55°C for 20 s, extension at 72°C for 5 min and a final extension step at 72°C for 7 min. PCR products were purified with GenElute PCR Clean-Up Kit (Sigma) and quantified using Qubit fluorometer (Invitrogen). Sequence reactions were performed using both MER 16s fwd and MER 16s rev primers with the GenomeLab DTCS Quick Start Kit (Beckman Coulter) following manufacturer's instructions. Sequence products were then purified using the Agencourt CleanSEQ kit (Agencourt) and capillary electrophoresis was accomplished using a CEQ 8000 Genetic Analysis System (Beckman Coulter). Raw data were analyzed using the Sequence Analysis software of the CEQ 8000 Genetic Analysis System suite v.9.0. Sequences were aligned using the AlignX tool in VectorNTI 7 (Informax Invitrogen).

RESULTS AND CONCLUSIONS - Amplicons of 430 bp have been sequenced in both forward and reverse strand to generate reliable consensus sequences. Sequences obtained from the four different species belonging to the Gadiformes order (*G. macrocephalus, G. morhua, M. aeglefinus* and *M. molva*) have been aligned and analyzed with AlignX of the VectorNTI suite (Informax Invitrogen) to discover SNPs which could differentiate them univocally. Three different SNPs, here identified with MerSNP-7, MerSNP-9 and MerSNP-1, have been shown to be able to generate aplotypes that allow the identification of the four species. Aplotypes were CCT, CCC, TAC and CAC for Pacific cod, Atlantic cod, Haddock and Ling respectively. (Table 1).

Table 1. Aplotype of the four different Gadiformes species.

	G. macrocephalus	*G. morhua*	*M. aeglefinus*	*M. molva*
MERSNP-7	C (G)	C (G)	T (A)	C (G)
MERSNP-9	C (G)	C (G)	A (T)	A (T)
MERSNP-1	T (A)	C (G)	C (G)	C (G)

Sequences obtained from 7 *G. macrocephalus,* 16 *G. morhua*, 15 *M. aeglefinus* and 5 *M. molva* samples collected at different fish catching campaign also confirmed our previous finding. A SBE (Single Base Extension) method is currently being developed for the three SNPs here discovered to allow an even more rapid and cost saving methodology for routine analysis as compared to re-sequencing. The 16s rRNA mitochondrial gene have been proved to be useful for authentication of seafoods products: it is present in higher copy number and abundance per sample extract than nuclear genes, moreover mitochondrial genome is uniparentally inherited avoiding sequence ambiguities from heterozygous genotypes and finally 16s rRNA gene, in particular, shows a relatively high point mutation rate which is very useful to identify even very closely related species. The design of interspecific conserved primers among different species belonging to the Gadiformes order has also been shown to be a useful tool to amplify common 16s rRNA region, thus extending very likely the number of species that could be analyzed with the same protocol that are interested in genetic traceability for economically or legally issues.

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