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Short communication

Forensic DNA analysis reveals use of high trophic level marine fish in commercial aquaculture fish meals

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ABSTRACT

Employing a short fragment of the mitochondrial 16S rDNA as a molecular tool we have analyzed commercial samples of three types of fish meal employed to feed aquarium cichlids, farmed salmonids and aquarium marine fish in Spain. We have identified a minimum of eight different marine fish species in their composition, all of them predators belonging to high trophic levels. Although some of them could be derived from the fish processing industry, the origin of at least two species is probably from extractive fisheries. These results suggest that more efforts are necessary for replacing fish protein in commercial fish meals in order to minimize the risks of feeding farmed fish with wild fish.

1. Introduction

Fisheries drive drastic changes in marine ecosystems. They cause diversity losses (Tisdell, 2003), as well as shifts in mean trophic levels that are declining systematically since 1950 (Pauly, 1998) because fisheries preferentially target predators, whose populations are declining as a consequence (Myers and Worm, 2003, 2005). Cascade effects occur subsequently along the trophic chain, as scarcity of top predators alters the normal regulation of their prey (McQueen et al., 1986; Hunter and Price, 1992; Verity and Smetacek, 1996; Daskalov, 2002) and breaks the equilibrium of the whole ecosystem.

Aquaculture was initially considered an ecological-friendly way of supplying fish protein for human nutrition because it was expected to reduce fishing pressure (Tidwell and Allan, 2001). However, consumers prefer carnivore species like salmonids, turbot, cod and others, which are highly prized and become preferential species for aquaculture. Cultured carnivore fish require in turn high-quality protein for their own development (Novell, 1991), and tons of protein-rich meals are required for feeding farmed fish. Overfishing can thus be promoted by aquaculture when such protein is obtained from extractive fisheries (Hannesson, 2003), becoming an enormous risk for marine ecosystems (Pauly, 1998; Naylor et al., 2000).

To minimize the impact of using fishery production for feeding farmed fish, fish meals in aquaculture diets are progressively substituted by plant proteins (e.g. Gomes et al., 1995; Allan et al., 2000) and also meat proteins (e.g. El-Sayed, 1998; Allan et al., 2000; Millamena, 2002). However there is evidence that fish species are still employed for aquaculture feeding purposes at large-scale in some countries like Vietnam (e.g. Edwards et al., 2004). In Europe, aquaculture is seen as a risk for ecosystems due to environmental impacts like pollution derived from aquaculture wastes (reviewed by Read and Fernández, 2003), interactions between farmed and natural populations through disease spreading (e.g. Naylor, 2005) and introgression of farm escapes in the wild (e.g. McGinnity et al., 2003; Bekkevold et al., 2006). However, the possible impact of feeding million of tons farmed fish with wild fish has not been analyzed in detail until now. It is generally assumed that fish meals employed in Europe may contain sub-products of seafood industry (bones, spines, heads) from species that are sold processed. In Spanish legislation there is a Royal Decree (465/2003) that regulates undesirable substances in products intended for animal feed and the maximum levels allowed of undesirable substances in them, to protect human health, animal health and the environment. However, the analysis and control of species contents in fish meals employed in aquaculture are not routinely carried out in Spain.

The purpose of the present study was to analyze dry commercial fish feed used to feed three different types of aquaculture fish: salmonids, marine species, and aquarium cichlids. The objective was to identify the species present in their composition by mean of DNA analysis, and assess the risk posed by aquaculture to aquatic ecosystems derived from the use of wild fish for producing fish

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feed. For this task we have employed a small fragment within the 16S rRNA gene. Although there are many projects for fish species identification based on sequencing of different genetic targets and longer amplicons, like barcoding projects (Hebert et al., 2003), in food science, DNA can suffer fragmentation due to the processing technologies applied to certain foodstuffs. In the case of fish meals, they are subjected to high temperature processes and high pressure to facilitate the grinding of their components. In those cases PCR amplification could fail, especially when the primers define DNA fragments of high molecular weight (Matsunaga et al., 1999; Frezza et al., 2003). Therefore, in samples where the level of DNA degradation is expected to be high, it is preferable to amplify smaller DNA fragments (Hird et al., 2003; Rodríguez et al., 2004; Arslan et al., 2006), such as the internal fragment of 16S rRNA analyzed in this work. The sequences will be compared against the GenBank for identifying the species. Although the GenBank contains errors (e.g. Harris, 2003), we choose for comparison only entries with clear specifications of voucher specimens, to be reasonnably sure about the identification. The 16S rDNA is one of the most sequenced mitochondrial regions, and is a reference for forensic identification of vertebrate species (e.g. Kitano et al., 2007). Therefore many 16S rDNA sequences can be found in the GenBank from most commercial fish species, allowing higher resolution level in case of intraspecific polymorphisms.

2. Materials and methods

2.1. Sampling

Three different types of fish meal were analyzed, employed for feeding fish in diverse sectors of aquaculture in Spain: freshwater (Salmonids), marine (Sparidae) and aquarium (freshwater fish cultured in recreational and domestic aquariums). The fish meal employed in aquariums was specifically sold for feeding herbivorous cichlids, as clearly stated on the label. Aliquots of five commercial samples of each type of aquaculture fish meals were directly provided by Spanish producers, and the feed for aquarium cichlids was purchased from a pet shop. The presentation of the fish feed for cichlids were in dry pellets, and that of the two aquaculture feeds was granulated.

2.2. DNA extraction

DNA extractions from fish meal were carried out from 10 mg of commercial product. In the case of fish meal samples, it was necessary to eliminate the oil and fat content, because both components can interfere with the DNA extraction process. The degreasing process was carried out by resuspending the meals in a solution of methanol–chloroform–water (2:1:0.8) for 2 h followed by washing in distilled water, and finally PBS 1× buffer to eliminate the remains of the solution used previously. Total DNA was extracted with a method based on silica gel columns (QIAamp® DNA Mini Kit, Qiagen, Germany). The kit was used following the manufacturer's instructions. The tubes were stored at 4 °C or frozen at −20 °C for long-time preservation.

2.3. PCR amplification, cloning and sequencing

A fragment of the mitochondrial 16S rRNA gene was amplified by polymerase chain reaction (PCR), employing the primers 16S-HF (5′ATAACACGAGAAGACCCT3′), 16S-HR1 (5′ CCCACGGTCGCCCCAAC 3′) and 16S-HR2 (5′CCCGCGGTCGCCCCAAC 3′) developed by Horreo et al. (in press), which are submitted for patent (date 4 August 2010) with the Reference P201031217. The amplification reaction was performed in a total volume of 40 μl, including Promega (Madison, WI) Buffer 1×, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 20 pmol of each primer, 20 ng of template DNA, and 1 U of DNA Taq polymerase (Promega). PCR conditions were: denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 61 °C for 20 s and extension at 72 °C for 30 s, a final extension at 72 °C for 20 min and a post-extension at 20 °C for 1 min.

PCR products were visualized in 2% agarose gels with 3 μl of 10 mg/ml ethidium bromide. Stained bands were excised from the gel, and DNA was purified with an Eppendorf PerfectPrep Gel CleanUp® Kit prior to sequencing. After that, amplified and purified products were precipitated using standard 2-propanol precipitation and re-suspended in formamide. Fifty nanograms of DNA were sequenced using 5 pmol of each primer. Sequencing was performed with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) with BigDye 3.1 terminator system, at the Sequencing Unit of the University of Oviedo (Spain).

Sequences were visualized and edited employing the BioEdit Sequence Alignment Editor software (Hall, 1999). Sequences were aligned with the ClustalW application (Thompson et al., 1994) included in BioEdit.

The fish meals contained more than one species and clean sequences could not be obtained directly because they cannot be properly distinguished in a chromatogram. As the chromatogram revealed species mixture, the PCR amplification products were cloned and 20–25 clones sequenced from each fish meal compound. The cloning kit employed was pETBlue-1 AccepTor™ Vector Kit, Novagen, following the manufacturer's instructions. Briefly, purified PCR products were linked into the plasmid pETBlue™-1 AccepTor™ in a tube with 1 μl of the vector (50 ng/μl), 4 μl of the PCR product (approximately 150 ng of DNA) and 5 μl of the cloning pre-mix provided by the manufacturer. The reaction mixture was incubated at 16 °C for 2 h. Then 1 μl of the mix was employed for transforming a 25 μl aliquot of competent *E. coli* cells (NovaBlue Single Competent Cells), with the following steps: at 0 °C for 5 min, at 42 °C for 30 s and further incubation on ice for 2 min. The transformed cells were resuspended in 250 μl of SOC medium, incubated at room temperature for 1 h, then enriched cells were transferred to Petri plates with LB agar (Luria-Bertoni) supplemented with 15 μg/ml tetracycline, 50 μg/ml carbenicilin, 70 μg/ml X-Gal and 80 μM IPTG, and incubated at 37 °C overnight. Petri plates were later placed in a fridge at 4 °C for 48 h for enhancing the color of colonies. Recombinant colonies (20–25 per fish feed) were selected for sequencing. They were resuspended in 50 μl of bidistilled water, incubated at 90 °C for 10 min for DNAase denaturing, and PCR amplified employing the primers pETBlueUp and pETBlueDOWN (1 μl of each 20 μM primer) in a reaction mix containing also 4 μl of PCR 5× Green Go-Taq Flexi Buffer (Promega®), 2 μl dNTPs, 2 μl $MgCl_2$ and 0.2 μl Go-Taq DNA polymerase 5 U/μl. The PCR products were separated by electrophoresis in 2% agarose gels and visualized by UV. Positive bands were excised and purified using the kit Wizard® SV Gel and PCR Clean-Up System and sequenced as described above, using the primer pETBlueUP (0.3 μl).

2.4. Identification of the species in sequence databases

Sequences obtained were compared with public databases (GenBank, http://www.ncbi.nlm.nih.gov/) employing the program BLAST within NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). BLAST finds regions of local similarity between sequences and can therefore be employed for identifying the species of unknown sequences, provided that sequences from voucher specimens of such species exist in public databases. Specifically, a Megablast search was carried out for evaluation of the degree of similarity between the sequences obtained using the new primers and those included in public databases. Sequence similarity >99% was considered reliable