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SHORT REPORT

Molecular identification of dolphinfish species (genus *Coryphaena*) using multiplex haplotype-specific PCR of mitochondrial DNA

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Abstract A rapid and reliable method of dolphinfish species identification was designed based on PCR amplification of diagnostic DNA fragments from the mitochondrial cytochrome *b* gene. It consisted in a tetraplex reaction producing a positive control amplicon and species-specific fragments in *Coryphaena hippurus* and *C. equiselis*. It was successfully tested in specimens of known identity and in nominal *C. hippurus* samples among which two *C. equiselis* were discovered. This approach has significant advantages over other molecular species identification methods and may help in determining species composition of mixed catches, and in forensic and food control applications of dolphinfish specimens or products.

Keywords Molecular species identification · Cytochrome *b* · MHS–PCR · *Coryphaena hippurus* · *Coryphaena equiselis*

Introduction

Dolphinfishes, *Coryphaena hippurus* (common) and *C. equiselis* (pompano), are widespread circumtropical epipelagic fishes of ecological and commercial relevance. They are sought after for their tackle in sport fishing and are also actively exploited commercially for their flesh, highly valued for human consumption (Herzig 1990). Annual catches vary widely geographically, from around 9,900 metric tons (mt) in Japan (Sakamoto and Kojima 1999) to 35 mt in the Mediterranean (Lleonart et al. 1999). In Latin America, dolphinfishes are exploited by artisanal fisheries (Arocha et al. 1999) and may represent a substantial fraction of long line catches (e.g., 75% in Costa Rica; Campos et al. 1993), which may reach 500 mt as in the Pacific coast of Colombia and Panama (Lasso and Zapata 1999). In Mexico, dolphinfishes are reserved by law for the sport fishing industry, and their commercial exploitation is prohibited. This situation generates some management challenges. They are fished as bycatch of long line and purse seine activities targeted at other pelagic species (Vaca-Rodriguez and Enriquez-Andrade 2002), and they are illegally fished due to their demand as seafood. Although illegal catches are elusive to assess, there are reports that they may represent as much as 55% of the artisanal finfish catches in the Mexican Pacific (Madrid and Beltran-Pimienta 2001). In either case, authorities are faced with the challenge of assessing the amount of unidentifiable dolphinfish products reaching seafood markets or restaurants, as their source is most likely illegal.

Unfortunately, there is a complete lack of information on the species composition of legal and illegal catches. It is thought that they comprise primarily *C. hippurus*; however, no estimates are available for the contribution of the apparently less abundant sympatric congener. Part of the problem lies in species identification. Even though pompano dolfinfishes are smaller, have a deeper body, longer pectoral fins and a differently shaped tooth patch on their tongue, these morphological differentiae are less conspicuous in

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young individuals, and *C. equiselis* can be easily mistaken for a young female of *C. hippurus*, particularly to the untrained eye. For example, seemingly unforeseen mixed catches of both species have been detected in the Canary Islands using allozyme electrophoretic variation, which revealed significant genetic differentiation between them (Pujolar and Pla 2002). This opens the possibility of developing a simpler genetic assay to help identify specimens of dolphinfishes and their products. Molecular methods to determine the taxonomic identity of biological samples have proliferated into a field of its own (Teletchea et al. 2005), particularly those based on DNA amplification by the polymerase chain reaction (PCR) due to their high sensitivity, applicability to minute or otherwise difficult samples, and the ease to scale up protocols with high throughput robotics. They have been used to identify a variety of samples, including undistinguishable early ontogenetic stages of fish (Hyde et al. 2005; Robertson et al. 2007; Rocha-Olivares 1998), or partially digested remains (Symondson 2002), and for ecological (Burton 1996), food control (Cocolin et al. 2000), and forensic purposes (Cronin et al. 1991).

In this paper we developed a sensitive and reliable method of identification of dolphinfish species, based on multiplex haplotype-specific (MHS) PCR that will be of value and directly applicable to address the following problems relating to their exploitation, commercialization, and consumption in Mexico and elsewhere: (1) determination of species composition of dubious specimens from legally landed catches, (2) forensic species identification applied to: carcasses found onshore or at sea and decommissioned illegal catches, and (3) food control of suspected products and mislabeling from seafood markets or restaurants.

Materials and methods

Rationale and primer design. MHS-PCR is a single-step identification of alternative DNA sequences based on the successful priming of the PCR mediated by complementarity of the 3′-end of a selective primer with diagnostic nucleotides of the target sequence. In addition, non-selective primers act as positive controls of amplification conditions (Rocha-Olivares 1998). Each sequence to be identified involves a different selective primer that in conjunction with one of the non-selective primers will produce an amplicon of diagnostic size. We used the mitochondrial (mt) cytochrome *b* (cyt*b*) gene for MHS-PCR due to the advantages of using mtDNA for fish species identification (Aoyama et al. 2000; Chakraborty et al. 2006; Robertson et al. 2007). We designed a pair of non-selective primers (L317 and H836, L refers to light-strand or forward primer and H to heavy-strand or reverse primer) producing a 554-base pair (bp) amplicon as a positive control of the reaction. Two primers, H678 specific for *C. hippurus* and H630 specific for *C. equiselis*, would each produce with L317 a species-specific amplicon of 397 and 352 bp, respectively (Fig. 1).

Experimental specimens. We used 95% ethanol fixed muscle samples of adult *C. hippurus* and juvenile *C. equiselis* to test and optimize the method. Specimens were positively identified by careful morphological determination. Common dolphinfish were from an Oahu Island, Hawaii, seafood market ($n = 3$) or fished offshore Mexico ($n = 5$), whereas pompano dolphinfish juveniles were collected by net trawling around the Hawaiian islands ($n = 7$). In addition, the optimized protocol was tested on a batch of 82 tissue samples collected from Chiapas, in the southern Mexican Pacific coast, originally identified as *C. hippurus* in the field.

DNA extraction. DNA was purified from tissues by standard proteinase K digestion and protein salting out (Aljanabi and Martinez 1997) and precipitated by centrifugation with chilled ethanol (100%). Quantity and quality of purified DNA were verified in a 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml).

MHS-PCR. Initially, each primer pair (L317-H630, L317-H678, L317-H836) was tested separately in gradient PCRs with annealing temperatures from 40 to 60°C to assess the stability of the reaction and to look for a compatible annealing temperature for all three reactions. Subsequently, tetraplex 13 μl MHS-PCR reactions were prepared using the forward (1) and reverse (3) primers: L317 (5′ A GGC CTG TAC TAT GGG TC 3′), H630 (5′ T TTT ATC GGA GTC AGA GTT TAG G 3′), H678 (5′ T AAA GCC AAG RAT GTC TTT G 3′), and H836 (5′ GG AAT TGA GCG GAG AAT TG 3′) (Fig. 1), by mixing 6.5 μl HotStart Taq Master Mix Kit (Qiagen, CA), 0.52 μl L184 (10.0 μM), 0.26 μl each H545, H497 and H703 (10.0 μM), 4.2 μl dH_2O (Quiagen) and ca 1 μl of purified DNA. The thermal cycling profile implemented in a PTC-200 (MJ Research) was as follows: initial hot start activation 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 52°C, and 60 s at 72°C; with a final extension of 5 min at 72°C. MHS-PCR products were verified in a 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml).

Results and discussion

Species typing and enhanced contrasts. Even though the annealing temperature of the primers ranged from 49.4 to 53.3°C, the optimization experiments showed that the reactions were very robust in the range of 40–60°C, obtaining single bands and no significant mispriming. Consequently, we opted for an intermediate annealing

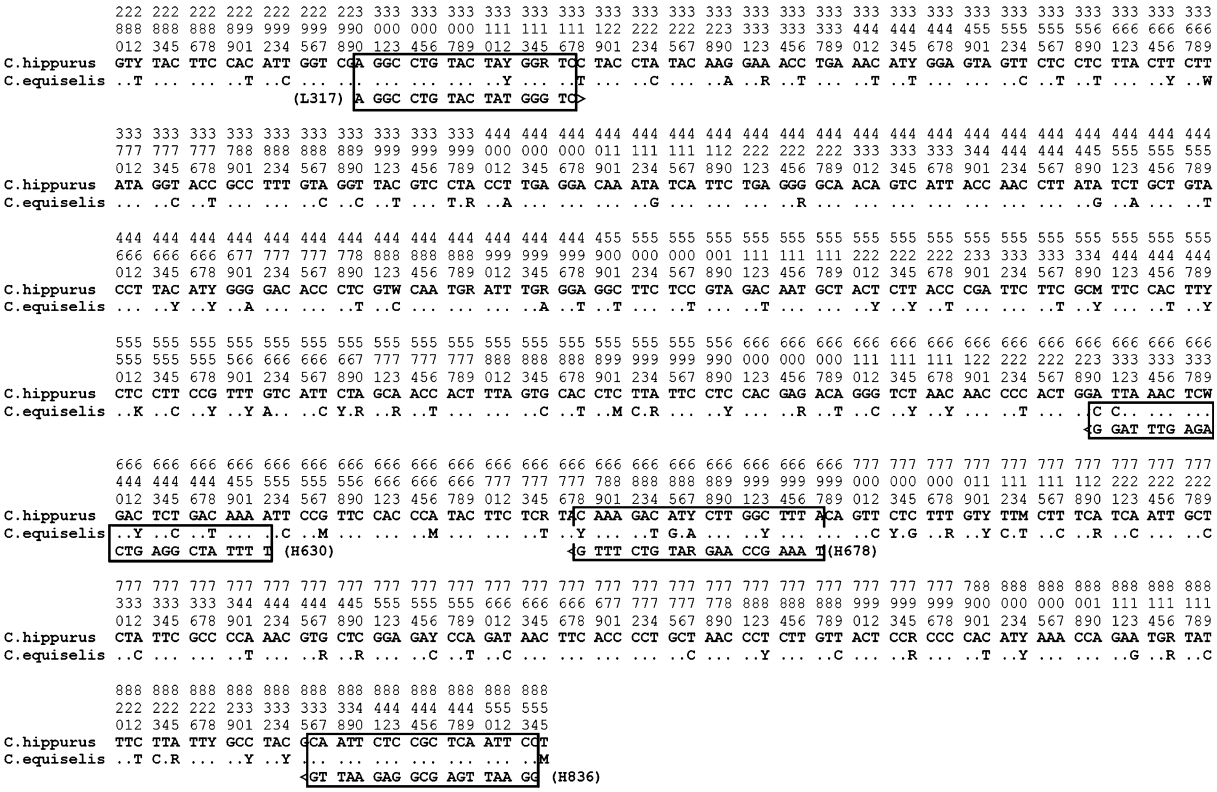

Fig. 1 Alignment of mitochondrial cytochrome *b* consensus sequences of *Coryphaena hippurus* (GenBank accessions: DQ197936, EF439513, EF439512, EF439196, AY895015, AY050761, and AY050762 from the Pacific, eastern and western Atlantic Oceans and the Mediterranean), and *C. equiselis* (GenBank accessions: L11533, AY89501, EF439194, and EF439195 from the Pacific and western Atlantic Oceans). Nucleotide positions are relative to the beginning of the gene, and each nucleotide triplet represents a codon. Primers used in the MHS-PCR design have been aligned to their homologous sequence, and their complementarity is indicated by boxes. See text for details. > forward primer, < reverse complementary primer

temperature of 52°C for the multiplex assay in which all three primer combinations performed appropriately (details not shown). Results of the MHS-PCR were as expected; all the positively identified *C. hippurus* specimens produced the predicted 554- and 397-bp bands, whereas *C. equiselis* amplified 554- and 352-bp bands (Fig. 2). In addition, the tetraplex reaction revealed an unforeseen difference between the *hippurus* and *equiselis* electrophoretic patterns reflecting a differential amplification of the bands depending of the template DNA present in the reaction. For the common dolphinfish, the species-specific band (397 bp) was faint and out competed by the larger non-specific band that appeared much brighter, whereas in the pompano dolphinfish both bands appeared equally intense, suggesting a lack of interference between amplicons. This amplification bias enhanced the differences between the electrophoretic patterns. MHS-PCR revealed that all but two of the specimens genotyped from Chiapas were correctly identified in the field as *C. hippurus*. Even though we cannot completely rule out the possibility of mislabeling during sample collection, we suspect that this was a case of misidentification given the small size of the specimens (52- and 49-cm fork length). We think this proves our case that mixed catches of both species may be more common than previously suspected.

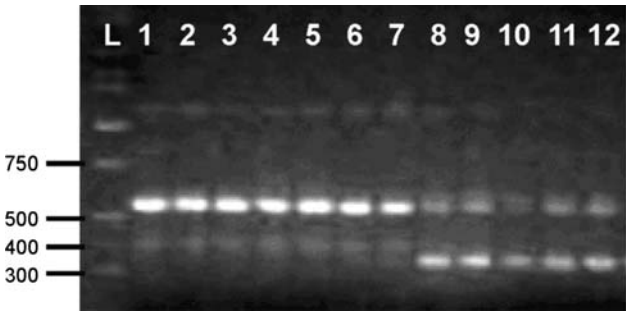

Fig. 2 Ethidium bromide stained 1.5% agarose gel showing the predicted amplicons from a MHS-PCR identification assay performed on positively identified samples of *Coryphaena hipurus* and *C. equiselis*. *L* DNA ladder, *lanes 1–7 C. hippurus*, *lanes 8–12 C. equiselis*. *Numbers* refer to the size (in bp) of DNA ladder fragments

Influence of intra-specific polymorphisms. The design of our MHS-PCR protocol was based on DNA sequences

of *C. equiselis* from the Pacific Ocean on which they were subsequently and successfully tested (Fig. 2). Recently, cyt*b* sequences of pompano dolphinfish from the North Atlantic (Madeira Archipelago) have become available (GenBank EF439195 and EF439194). These show that nucleotide 678 is polymorphic in *C. equiselis*, and North Atlantic sequences share a C residue with *C. hippurus*, see IUPAC Y(C or T) in the alignment of Fig. 1. Even though primer H678, specific for *C. hippurus*, has three other mismatched bases with the sequence of *C. equiselis* (Fig. 1), it is possible that the complementarity of the six 3′-end bp will be sufficient to generate some 397-bp amplicon. The dual specificity of primer H678, for *C. hippurus* and Atlantic *C. equiselis*, adds improved power to differentiate among *C. hippurus* (554- and 397-bp bands), Pacific *C. equiselis* (554- and 352-bp bands), and Atlantic *C. equiselis* (554, 397, and 352 bp bands), provided one of the mutations is fixed in each ocean basin, T in the Pacific and C in the Atlantic. We did not have samples of North Atlantic pompano dolphinfish to corroborate this prediction.

Comparison of molecular identification methods. MHS-PCR provides significant advantages compared to other PCR-based techniques designed for food product molecular species identification, such as PCR-RFLP (e.g., Cocolin et al. 2000) and RAPDs (e.g., Martinez and Yman 1999). It is simpler, less expensive, and less laborious than PCR-RFLP, which involves first amplifying the target gene in sufficient quantities followed by a gel check, and then restriction enzyme (RE) digestions of the PCR product followed by high-resolution gel electrophoreses. MHS-PCR obviates the use of RE since the DNA polymorphisms are detected by the PCR itself. MHS-PCR is more similar to RAPDs; however, it provides control over the nature of the assayed DNA polymorphisms that the latter anonymous loci cannot, resulting in improved power to address the issue of false positive or false negatives. The ability of MHS-PCR to predict and manage the consequences of newly discovered intraspecific polymorphisms affecting assays was made clear in the previous paragraph. An additional problem with RAPDs is the probability of non-homologous (homoplasious) positive alleles, resulting from convergence of similar sized allele amplification from independent loci. Ultimately, MHS-PCR shares with the rest of molecular species identification methods the same limitation, namely that its success depends on the existence of diagnostic mutations in the gene of interest for which we can design a detection method that is technically feasible and reliable.

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