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SNP-based PCR-RFLP, T-RFLP and FINS methodologies for the identification of commercial fish species in Egypt

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ABSTRACT

Genetic fingerprinting is important for both certifying authenticity and traceability of fish species. In the present study, three methodologies were developed for the identification of aquaculture and fisheries Egyptian fish: forensically informative nucleotide sequencing (FINS), single nucleotide polymorphism-based polymerase chain reaction-terminal restriction fragment length polymorphism (SNP-based PCR-T-RFLP) capillary electrophoresis, and SNP-based PCR-RFLP. FINS based on mitochondrial 12S rDNA allowed the genetic identification of seventeen Egyptian species analyzed. Four SNPs, located in restriction sites, also enable identifying all species. The resolution power of agarose gel electrophoresis was not enough to detect small-sized fragments, whereas T-RFLP capillary electrophoresis produced complete species-specific patterns. The three methods are ready now and can be employed as inexpensive, rapid and effective tools for food authentication in Egypt and other countries where seafood contributes much to local animal production economics.

1. Introduction

Egypt (22–32°N, 25–35°E) exhibits a great diversity of waters and water bodies in the Northeast of Africa. According to FAO (2010), Egypt has 2400 km of coastline in the Mediterranean and the Red seas. The total freshwater area in Egypt is estimated as 6000 Km^2, distributed mainly in the River Nile, its major two tributaries and many irrigation canals that flow through the country. Furthermore, several brackish and salty lakes are present, mainly Mariut, Edku, Manzala and Bardawil in the North; Qaroun in the Middle; and Timsah and Bitter Lakes in the North East. Another principal water body in Egypt is the greatest African artificial reservoir behind the Aswan High Dam, the Lake Nasser, which is a completely freshwater lake. In 2009, marine capture fisheries accounted for 127,821 tons, inland capture fisheries were 259,577 tons, and both were far less than aquaculture production that accounted alone for 705,490 tons. The fish groups that prevail the catch in Egypt, according to FAO fisheries statistics to 2009, are breams (4135 tons), groupers (3230 tons), emperors (2157 tons), and mullets (1524 tons), and mostly come from the Mediterranean fleets. Cultured fishes are mainly tilapias (390,000 tons), mullets (210,000 tons), and carps (74,000 tons) and catfishes (18,000 tons)-all of which are freshwater or brackish water fishes, together with the more brackish or marine fish species like the gilthead seabream and the European sea bass that contribute both the majority of the marine aquaculture industry in Egypt (5381 and 5335 tons in 2009, respectively) (FAO, 2010). Several other species are present in either the capture fisheries or aquaculture in Egypt, like sardines and catfishes. Yet, species like tilapias, mullets, breams, sea bass, groupers and emperors are the commonest ones in fish markets in both continental and coastal cities of Egypt.

Over the past decades, several protocols have been described for fish species identification due to the demand for reliable seafood traceability systems. Initially, protein-based methods such as ELISA and isoelectric focusing have been applied in the world of fish species discrimination (Asensio et al., 2008). Recent advances in molecular biology techniques, mainly the polymerase chain reaction (PCR)-based techniques, provided easy, cheap and reliable tools used in different fields of medicine and biology (Duran et al., 2009). Modern advance in such techniques are also contributing effectively to the certification of the species present in commercial

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Table 1
Numbers, collection sites and local names of voucher and commercial samples. GenBank accession numbers for the species added through the current study are also shown.

Voucher and commercial samples	n. voucher samples	n. commercial samples	Collection sites	Morphological & genetic assignments				
				Scientific name	Common name	Family	Egyptian local names	Accession numbers
Snappers	5	5	Hurgada city	*Aphareus furca*	Small toothed jobfish	Lutjanidae	EL Fares	KU680993
Emperors	20	20		*Lethrinus lentjan*	Pink ear emperor	Lethrinidae	El Sho'or	KU680994
				Lethrinus microdon	Smalltooth emperor	Lethrinidae	El Sho'or	KU680996
				Lethrinus harak	Thumbprint emperor	Lethrinidae	El Sho'or	KU680995
				Lethrinus mahsena	Sky emperor	Lethrinidae	El Sho'or	KU680997
Groupers	20	20		*Epinephelus tauvina*	Greasy grouper	Serranidae	Waqar	KU680998
				Epinephelus polyphekadion	Camouflage grouper	Serranidae	Waqar	KU680999
				Anyperodon leucogrammicus	Slender grouper	Serranidae	Waqar	KU681000
				Cephalopholis sonnerati	Tomato hind	Serranidae	Nagil	KU681001
Tilapias	10	10		*Oreochromis niloticus*	Nile tilapia	Cichlidae	Bolti	KU681002
				Tilapia zillii	Green tilapia	Cichlidae	Bolti	KU681003
Mullets	10	10	Kafr El-Sheikh	*Mugil cephalus*	Flathead grey mullet	Mugilidae	Bory	KU681004
				Liza ramada	Thinlip grey mullet	Mugilidae	Tobar	KU681005
Carps	10	10		*Cyprinus carpio*	Common carp	Cyprinidae	Mabrouk	KU681006
				Hypophthalmichthys molitrix	Silver carp	Cyprinidae	Mabrouk Feddi	KU681007
Sea bass	5	5	Damietta	*Dicentrarchus labrax*	European seabass	Moronidae	Qaroos	KU681008
Sea bream	5	5		*Sparus aurata*	Gilthead seabream	Sparidae	Denis	KU681009

food products. The use of specific marker genes for identification of component species in foods is of special importance in this sense (Perez et al., 2005).

The major advantage of the DNA-based methodologies is that they provide accurate identification of finfish and shellfish species even in severely processed food stuffs (Ardura et al., 2010; Galal-Khallaf et al., 2016; Rasmussen and Morrissey, 2008). In general, short and informative genomic sequences (barcodes) are now widely applied for species identification for various purposes, including market traceability (Ardura et al., 2010; Galal-Khallaf et al., 2016). Principally, the mitochondrial cytochrome oxidase subunit I (COI) has been used as the universal system for barcoding (e.g. Galimberti et al., 2013). Other mitochondrial genes were also used for barcoding. The 12S rRNA gene was reported as a good marker for authentication of fish and seafood based on its acceptable length, low mutation rate, sufficient interspecies differentiation and less degeneration than the mitochondrial protein-coding genes (Cespedes et al., 2000; Comesaña et al., 2003; Rasmussen and Morrissey, 2008). A broad spectrum of fish orders can be successfully identified through the 12S rRNA gene, including Characiformes, Osteoglossiformes, Perciformes, Pleuronectiformes, Siluriformes (Ardura et al., 2010; Comesaña et al., 2003).

More recently, single nucleotide polymorphism (SNP) is a promising molecular marker in genetics, genomics and aquaculture (Zhu et al., 2012). SNPs are becoming almost the dominant molecular marker because they are: i) abundant in all genomes; ii) rapid and efficient assays available for genotyping; and iii) with low mutation rate, what makes them stable and do not change significantly from generation to generation (Duran et al., 2009; Garvin and Gharrett, 2007; Pérez et al., 2004).

One simple, inexpensive, quick and accurate laboratory technique for SNP genotyping is polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) that requires minimal investment in instrumentation (Ota et al., 2007). In PCR-RFLP analysis, PCR-amplified DNA is recognized and endonuclease digested in the region of the point mutation (SNPs sites), and the fragments generated are separated by agarose gel electrophoresis. However, the conventional PCR–RFLP gel-based method cannot detect small restriction fragments due to its low resolution (Wang et al., 2010). Instead, terminal restriction fragment length polymorphism (T-RFLP) approach is based on the amplification of a specific gene using fluorescently labeled primers, and the restriction fragments are separated and detected by capillary electrophoresis with laser-induced fluorescence detection (Layer et al., 2007). Capillary electrophoresis has much more precise and accurate detection for DNA fragments compared to agarose gel electrophoresis, and can easily discriminate fragments of similar size. The T-RFLP capillary electrophoresis methodology in combination with SNPs located in restriction sites also proves to be useful to identify coffee (Spaniolas et al., 2006), olive oils (Bazakos et al., 2012) and fish species (Dooley et al., 2005b). This method has not been applied to the recognition of Egyptian fish to date.

Various genetic fingerprinting studies have been applied for Egyptian fish species discrimination (Abdel-Hamid et al., 2014; Awwad and Badawy, 2007; El-Serafy et al., 2003; Mohammed-Geba et al., 2016). However, they are not used in routine seafood controls because they have been developed only for a few species (Tilapia, *Bagrus* spp.). Genetic barcoding has also proven to identify few more species such as *Pangasianodon hypophthalmus* and *Oreochromis niloticus* (Galal-Khallaf et al., 2014). The objective of the present study was to develop efficient molecular markers for identification and characterization of the most important Egyptian aquaculture and fisheries fish species, for use in fish market traceability in Egypt. We characterized a number of SNPs and used those located in restriction sites. To our knowledge, SNP based-T-RFLP and FINS methodologies underwent for the first time for genetic identification of Egyptian fish species.

2. Materials and methods

2.1. Sampling

2.1.1. Voucher samples

In order to represent the most important fisheries and aquaculture fishes in Egypt, 170 specimens, representing the main 17 commercialized fish species, were collected from three Egyptian governorates, that are the Red Sea, Kafr El-Sheikh, and Damietta. Their scientific and common names, as well as the locality of sampling, are in Table 1. Forty five specimens of snappers, emperors and groupers from Hurgada city (the Red Sea Governorate, marine fisheries) and thirty samples of tilapias, mullets and carps from Kafr El-Sheikh Governorate, freshwater aquaculture species), were collected (Table 1). In addition, five samples for each seabass and seabream were collected from El-Deeba Triangle (Damietta Governorate, marine aquaculture species). All specimens