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Development of cytochrome-c-oxidase 1 specific primers for genetic discrimination of the European eel *Anguilla anguilla* (Linnaeus, 1758).

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Abstract
The European eel *Anguilla anguilla* (Anguillidae, Anguilliformes) is amongst the most peculiar, economically-important fish species in the Egyptian and Mediterranean waters. Very rare studies are available about its populations in the Mediterranean. In order to investigate this issue, we designed cytochrome-c-oxidase subunit 1 (COI) gene-specific primers for *A. anguilla*. These primers targeted the amplification of a 750 bp fragment towards the 5` extremity of COI. The primers were tested for *A. anguilla* elvers collected from Rachid Estuary in Egypt. They succeeded to amplify the targeted fragment. Using Forensically Informative Nucleotide Sequences (FINS) analysis and BLAST comparisons, the sequences were successfully assigned to *A. anguilla*, with clear segregation from other *Anguilla* species. The primers designed and used in this study can be then suggested as successful alternatives for universal primers commonly used in European eel barcoding.

Keywords: *Anguilla anguilla, CO1, FINS, molecular markers, population genetics, primers.*

1 Introduction

The European eel *Anguilla anguilla* (Anguillidae, Anguilliformes), inhabits a wide range of climatic habitats, being found in all rivers from Northern Norway and southwards along the Western coasts of Europe to almost all coasts of the Mediterranean Sea but Libya where no rivers exist naturally (Schmidt 1909, Dekker 2003). It occurs widely in most inland waters of Europe (e.g. lakes) (Defra 2010; Jacoby and Gollock 2014).

A. anguilla is categorized as ¨catadromous¨ species, where survival of the fish is in freshwater, but it migrates for spawning in seawater. *A. anguilla* starts puberty, turns from dark olive color to silver one (i.e. silvering), leaves its feeding beds in the coastal lakes and rivers to the Mediterranean water for the onset of migration. Migration starts in late summer, beaks during the autumn, and declines in the middle of the winter (Han et al., 2001). Fully maturing into adults upon migration, adults spawn in the deep waters of the Saragsso Sea (in front of the eastern US coast). Adults die as their digestive systems suffer degeneration and atrophy that gradually proceed to the maximum with sexual maturation completeness (Pankhurst and Sorensen, 1984; Larsen and Dufour, 1993). The eggs hatch on leaf-like leptocephali larvae that disperse and migrate to metamorphose into glass eels after several months of drifting. Glass eels become pigmented elvers in the estuaries and enter fresh or brackish waters to grow until silvering, the major preparation for spawning migration.

The European eels have several life history characteristics that make them particularly vulnerable to overexploitation: they are large, live long, mature late, produce all their offspring at once, labile to heavy mortality, and migrate long distances. There is significant international trade demand for the species, both for live glass eels (from Europe to Asia) and the highly valued meat of adults (Maes and Volckaert 2007). Adult eels (yellow and silver stages) are intensively exploited in North European and Mediterranean countries (Dekker, 2000, 2003). The recruits of the species (glass eel and elver stages) are harvested commercially during their mass migration to the continental coast. The majority of glass eels catches is used for aquaculture in Europe and in eastern Asia and for restocking purposes (mainly in northern Europe) (Zompola et al., 2008). Due to rapid development of the eel aquaculture industry in the Southeast Asian countries, elvers in the estuaries were over exploited, which have severely influenced the recruitment of the eel in the rivers (Han et al., 2001).

A. anguilla numbers have dropped as much as 99% since the early eighties of the previous century (Dekker, 2003). Wild stocks of the European eel, *A. anguilla*, are no longer considered sustainable, with levels of glass eel returning currently between 1 and 9% of those reported in the 1970s and 25 % of yellow and silver eels as reported by Food and Agriculture Organization of the United Nations(FAO) in the 1960s (Dekker, 2003; ICES 2008, 2010). The European eel is thus listed as a critically endangered species on the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species and has been added to the Annex B of the Convention on International Trade in Endangered Species (see Freyhof and Kottelat, 2008; Maldonado et al., 2011).

DNA barcoding is a new approach in the study of the genetics where a short, standardized region of certain gene is used for species characterization. The commonest gene for this kind of studies is the mitochondrial cytochrome-c-oxidase 1 gene (COI). The ¨barcoding¨ region shows high inter-species but low intra-specific divergence, allowing then species and populations' discrimination (Hajibabaei et al., 2006; Hebert et al., 2003a, 2005a; Pappalardo et al., 2015). Since no information is available about the Egyptian populations of *A. anguilla*, we aimed by this study to isolate, amplify, and sequence the partial barcode regions of the gene COI in *A. anguilla* as a preliminary step towards its molecular fingerprinting. Furthermore, we applied the forensically informative nucleotide sequencing (FINS) methodology and the phylogenetic relationships to identify the correlation between the studied regions in the COI gene of *A. anguilla* and the same region in other *Anguilla* species.

2 Materials and Methods

2.1. Designing of COI-specific primers

Twenty *A. anguilla* full mitochondrial genomes were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/?term=Anguilla+anguilla+mitochondrion). All these sequences were aligned using ClustalW algorithm (Thompson et al., 1994) integrated to Mega6 (Tamura et al., 2013). The sequences were then trimmed to obtain a final common zone of a 3,000 base pairs-long gene fragment, representing full COI gene of *A. anguilla*. A single sequence was uploaded to primer3plus algorithm (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) in order to design a pair of primers with annealing temperature between 50-60 ºC, maximum repetitions of the same nucleotides less than 3, and expected amplicon size between 700- 1000 bp in the hypervariable region of the COI gene in fishes, that is the 5´ area used before by other authors (e.g. Ward et al., 2005). Other criterion in the primer design, that was manually checked regardless to the program primer3plus, is the least self complementation of bases in each given primer alone and between the two primers in the selected primer pair (not exceeding 3 complementary bases adjacent to each other) for avoiding formation of hair-pin loops.

2.2. Sampling and samples preservation

Ten (n=10) *A. anguilla* elvers (10±5 cm total length) were collected from landings in Rachid City (Rosetta), Egypt. The samples were transferred to the laboratories of Genetic Engineering and Molecular Biology in the Faculty of Science of Menoufia University (Shebeen El-Kom City, Egypt). They were anesthetized using commercial clover oil. About 100 mg of the caudal fin of each sample were dissected and stored in absolute ethanol until DNA extraction. All the individuals were stored in -20 ºC until further analysis.

2.3. DNA purification and COI-based polymerase chain reaction (PCR)

Total genomic DNA was purified from 15 mg of caudal fin of *A. anguilla*, using the method for DNA extraction from fish fins and other tissues as in Wasko et al. (2003) and modified by Mohammed-Geba et al. (in press) as it well be mentioned. All concentrations found herein are the final concentrations; otherwise any modification will be mentioned. Fin clips were lysed individually using 200 µL of TNES-urea buffer (See Wasko et al., 2003) and 2.4 U mL^{-1} Proteinase K solution (ThermoFischer Scientific), with incubation at 55 ºC for 30 min. Later on, 54 µL of 5 M NaCl were added, the tubes were thoroughly mixed by inversion, then centrifuged at 4,000 g for 10 min. The supernatant from each sample was transferred to other 1.5 mL eppendorf tube, and the DNA was then precipitated by adding 200 µL of cold isopropanol (at -20 ºC) with shacking by inversion. The tubes were centrifuged at 11,000 g for 10 min, and the supernatant was completely removed. The DNA pellet was washed by 400 µL of 70 % Ethanol, centrifuged for 5 min at 11,000 g and poured completely from ethanol, then 30 µL of Tris EDTA buffer (10 mM TRIS.HCL pH8, 2 mM EDTA pH8 and 19.720 µL of sterile double distilled H_2O) were added for DNA pellet resuspension. DNA quality was checked by running 5 µL of the genomic DNA with 1 µL of 6x DNA loading buffer (0.25 % w/v bromophenol blue, 40 % w/v sucrose), in 1 % agarose gels stained by 0.5 µg mL^{-1} ethidium bromide (ThermoFisher Scientific). DNA samples were used directly for amplification of partial barcode region of the COI gene by PCR.

Specific *A. anguilla* forward and reverse primers were used for amplification of COI gene in all samples. The amplification reactions were performed in a total volume of 50 µL. The reaction mixture consisted of 2 µL of template DNA (~50 ng), 0.5 µM of each primer, 25 µL of 2 x of MyTaq red master mix (Bioline), and completed to 50 µL with PCR-grade water. PCR amplifications were done in the thermal cycler *Tpersonal48* (Biometra, Germany). PCR program contained an initial denaturation step at 95 ºC for 5 min, followed by 40 cycles of 95 ºC for 30s, 56 ºC for 1 min, 72 ºC for 1 min, and a final extension of 72 ºC for 10 min. PCR product were electrophoresed in a 1 % agarose gel. Adequate sized PCR products were sent to Macrogen Inc. (Seol, South Korea) for sequencing.

2.4. Sequence analyses and FINS-identification of eel elvers

The quality of the obtained sequences was manually checked with the freeware Chromas Lite 2.1. Sequences were manually checked and corrected for

nucleotide deletions/insertions whenever necessary. Each sequence was individually compared to GenBank database using basic local alignment search tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi: Altschul et al., 1990). Up to five COI sequences from each species belonging to the genus *A. anguilla* were retrieved from the GenBank database in order to construct the phylogenetic tree used for species confirmation. All sequences were then aligned using CLUSTAL W Multiple alignment implemented in Mega6 software, as mentioned before. Obtained *A. anguilla* COI sequences were translated into the primary amino acids sequences (Figure 2) using vertebrate mitochondrial codons in Mega6 in order to check for the absence of COI nuclear copies (NuMTs). Best nucleotide substitution model was selected using ModelTest algorithm implemented to the program Mega 6. Then, a Neighbor-Joining phylogenetic tree was constructed using 1,000 bootstraps (tree replicates), in order to infer the phylogenetic relation of the obtained Egyptian samples of the European eel and the other eel available in the GenBank database.

3 Results

Full mitochondrial sequences aligned shared maximum intra-specific nucleotide similarity. Figure 1 shows an example for the alignment and the conserved sites in *A. anguilla* full mitochondrial genomes. Primer pair that complied with the criteria mentioned above is shown in Table 1.

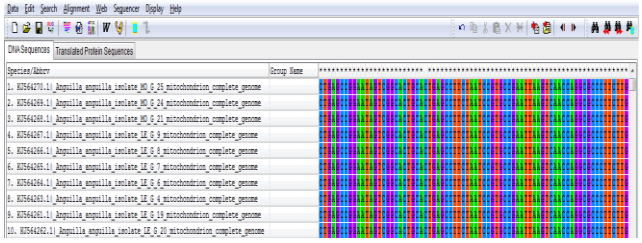

Figure 1. Part of full mitochondrial genomes of *A. anguilla* aligned after retrieval from GenBank database.

Table 1. Sequences of primers used for amplification of partial 5′ fragment of Egyptian *A. anguilla*samples.

Primer designation	Primer Sequence (5′→3′)
*A. ang*Fw-1	CTGCAYTGAGCCTTCTAATC
*A. ang*Rv-1	GATAATTATTGTGGCGGAAG

A 757-bp fragment of the COI gene could be amplified by PCR, as visualized by the agarose gel electrophoresis (Figure 2). Sequencing resulted in 100 % sequence identity with published *A. anguilla* sequences in the GenBank database. The sequences were submitted to GenBank database under accession numbers KU980202-KU980211.

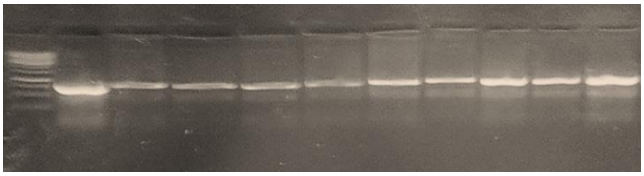

Figure2. 1 % agarose gel electrophoresis showing the results of amplification of *A. Anguilla* partial COI gene by the designed specific primers. Ladder bands scale (left): 250 bp-500 bp-750 bp-1000 bp-1250 bp-1500 bp-1750 bp-2000 bp.

Comparing the obtained sequences to these of the full mitochondrial genomes of *A. anguilla*, and the fragment obtained by the common DNA barcoding protocol of Ward et al. (2005), the sequences of the PCR products obtained from our primers overlapped with these obtained by Ward et al. (2005) in almost 74 % of Ward´s common fragment in the 5` region of the COI gene in *A. anguilla* (Figure 3).

For FINS identification of our samples, the best substitution model to describe our data with the retrieved GenBank database sequences was Tamura-three parameters, with Gamma value of 0.25. The constructed phylogenetic tree showed prominent results in what concerns the position of our samples within other *A. anguilla* vouchers presenting in the GenBank database, and the position of different *Anguilla* spp. together in relation to their geographical proximity. All Southern hemisphere *Anguilla* spp. clustered together, while all Northern hemisphere species clustered together-both with bootstrap values high enough to support the accuracy of the test and ,consequently, to confirm their proximity (Figure 4).

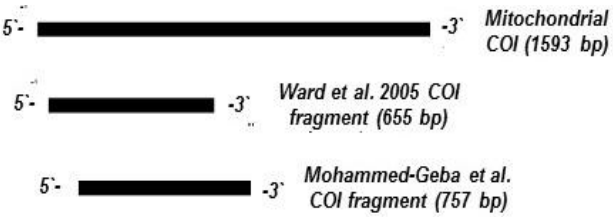

Figure3. Diagrammatic view for the common region in the COI gene sequence amplified by PCR primers of Ward et al. (2005) and the ones applied in the current study, in comparison to a full mitochondrial COI gene sequence (acc. no. KJ564270.1).

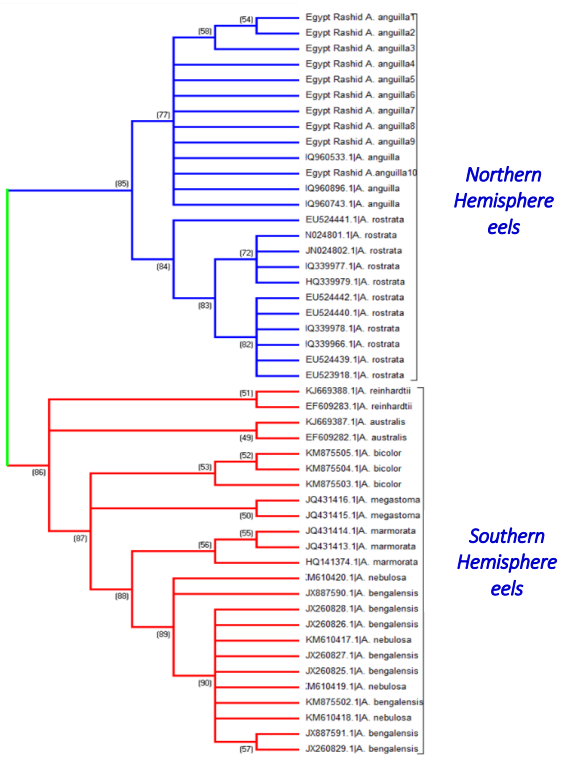

Figure4. Neighbor-joining consensus bootstrap phylogenetic tree constructed for different *Anguilla* spp. Numbers on branches refer to bootstrap values. Blue color: Northern Hemisphere eels, Red color: Southern Hemisphere eels.

4 Discussion

The primers designed could efficiently characterize *A. anguilla* through PCR-based amplification of the COI gene. We used this approach as a result of preliminary trial for use of common barcoding protocol by Ward et al. (2005), but resulted in some degree of sequence overlapping. Hence, we passed to the design of more gene- and species-specific COI primers. For*Anguilla* spp. discrimination, Ward et al. (2005) primers showed good success, for instance for monitoring the occurrence of *A. bengalensis bengalensis* and *A. bicolor bicolor* in Peninsular Malaysia (Arai et al., 2015), for ichthyfauna species identification in Turkey, including *A. anguilla* (Keskın and Atar, 2013), and for characterization of freshwater fishes in Germany (Knebelsberger et al., 2015).

FINS assigned the resulting sequences successfully to the targeted species. Clustering of *A. anguilla* and *A. rostrata* together in the same clade but two sister subclades coincided well with the known mitochnodnrial genome-based phylogeny of these two species as reciprocal monophyletic lineages (Jacobsen et al., 2014).Since its description by Bartlett and Davidson (1992), FINS methodology for fish species identification and authentication was used successfully. It relies mainly on the least pairwise differences and/or the least nucleotide substitution between the tested samples and the other ones available in the genetic database (Bartlett and Davidson, 1992; Rasmussen and Morrissey, 2008). For examples, COI-based FINS could successfully characterize *Scomber scombrus*, *S. australasicus*, and *S. japonicus* (Perciformes, Scombridae) both as fresh and canned products. Flatfishes of the species *Pleuronectes platessa*, *Solea solea*, *Hipoglossus hipoglossus*, *Reinharditus hippoglossoides*, *Limanda ferruginea*, and *Microstomus kitt* could be enough separated by such methodology. Even for cartilaginous fishes, different shark species in the Egyptian Mediterranean waters, namely *Squalus acanthias*, *Oxynotus centrina*, *Squatina squatina*, *Scyliorhinus canicula*, *Scyliorhinus stellaris*, *Mustelus mustelus*, *Mustelus punctulatus* and *Carcharhinus altimus*, could be efficiently discriminated by COI-based FINS (Moftah et al. 2011).

Therefore, and for the first time in Egypt, we provided accurate method for *A. anguilla* species discrimination through designing of COI gene-specific primers and FINS methodology. This work opens wide horizons for investigation of ecogenetics of *Anguilla anguilla* in Egypt and the world.

5 References

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