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Method paper

A molecular approach towards taxonomic identification of elasmobranch species from Maltese fisheries landings

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ABSTRACT

The mitochondrial genome, through the application of DNA barcoding, provides a powerful tool for identifying species even when specimens are either incomplete or belong to species that exhibit cryptic diversity. In fisheries management accurate identification of whole or part of the specimens landed is a fundamental requirement for the conservation of species affected directly or indirectly by the fisheries activities. In this study cytochrome *c* oxidase subunit I (COI) and NADH dehydrogenase subunit 2 (ND2) sequences were used to genetically distinguish 36 elasmobranch species collected from Maltese (Central Mediterranean) commercial fisheries landings. Each species was analysed using these two mtDNA loci where COI (610 bp) and ND2 (990 bp) efficiently distinguished between the various species studied, leading to the identification of 101 haplotypes, with the intraspecific *p*-distance ranging between 0 and 0.75% (mean 0.10%, SD ± 0.13%). This study enhances the molecular data available on elasmobranchs by providing new ND2 sequences for various species, while providing both COI and ND2 data for poorly studied Mediterranean species including: the large pelagic sharks *Alopias vulpinus*, *A. superciliosus*, *Carcharhinus altimus*, *C. plumbeus*, *Carcharadon carcharias*, *Isurus oxyrinchus*, *Prionace glauca* and *Odontaspis ferox*; the smaller demersal sharks *Somniosus rostratus*, *Squatina aculeata*, *S. oculata* and *Squalus* sp.; and the endemic stingray *Dasyatis tortonesei*. It also confirmed the landings of species whose identification relies strongly on molecular tools, namely *Squalus* sp. and *D. tortonesei*, which are both first confirmed records amongst Maltese fisheries landings. Morphologically, the latter two species, can be easily misidentified with *S. blainville* and *D. pastinaca* respectively. Additionally, this study evaluated the genetic differences between different polychromatic forms of *Raja clavata*, *R. radula* and *Dipturus oxyrinchus*. Based on the currently analysed specimens, no significant genetic differences were found between the various forms and thus no further speciation within the species was identified.

1. Introduction

The Mediterranean Sea is estimated to host around 86 species from the subclass Elasmobranchii (Compagno, 1984a, 1984b; Serena, 2005; Bradai et al., 2012; Froese and Pauly, 2016). At Mediterranean level they represent the most vulnerable group of fish species, with some of them being in a worse regional conservation status when compared to their global conservation status (Abdul Malak et al., 2011; IUCN, 2017).

While in various regions around the world, elasmobranchs are generally exploited for their meat, fins, leather, liver oil, cartilage, fishmeal, teeth and jaws (Rose, 1996; Bradai et al., 2012), in Malta these species are solely sold for meat consumption, with the majority of the species being caught as by-catch of teleost fisheries (Dalli, 2004; Vella and Vella, 2010; observations by authors). Due to this, some species of Elasmobranchii are rarely represented in the Maltese landings, while others such as *Hexanchus griseus*, *Mustelus* species, *Squalus* species, and *Raja clavata* are economically important and are frequently caught as primary or secondary target species by a limited number of Maltese small-scale fishermen who alter their fishing gear according to season (Dalli, 2004; Vella and Vella, 2010; observations by authors). To safeguard these small-scale fisheries, correct species identification is a basic requirement for biological data collection and for species specific monitoring, ensuring sustainable fisheries management in the area (Vella, 2009; Vella et al., 2016; Vella and Vella, in press). FAO data for the Mediterranean basin indicates that a number of elasmobranch species are clustered in unidentified categories, while others are classified down to high taxonomic levels such as Squalidae or Dasyatidae (FAO, 2017).

For accurate genetic identification, this study utilized the conventional cytochrome *c* oxidase subunit 1 gene (COI) (Ward et al., 2005, 2008; Moftah et al., 2011; Schembri, 2013; Landi et al., 2014; Verissimo et al., 2014; Vella et al., 2016; Cariani et al., 2017; Karahan

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et al., 2017) and the faster evolving NADH dehydrogenase subunit 2 gene (ND2) (Moore et al., 2011; Naylor et al., 2012; Last et al., 2016), as the combination of these two mitochondrial DNA (mtDNA) genes has proven to be reliable in discriminating between different elasmobranch species (Moore et al., 2011; Last and Seret, 2016; Verissimo et al., 2016). This study covers a number of regionally Vulnerable, Endangered and Critically Endangered species (Supplementary data - Table 1) whose occurrence in the central Mediterranean is poorly known, with some of them being only recorded as rare sporadic landings.

Molecular systematic studies of elasmobranch species have led to the identification of new species and the resurrection of taxa (Naylor et al., 2012; Vella and Vella, 2013; Last et al., 2016; Verissimo et al., 2016). Recent works have led to changes in nomenclature of *Dasyatis centroura* to *Bathytoshia centroura* (Last et al., 2016; IUCN, 2017) and *Centrophorus granulosus* of Mediterranean origin to *C. uyato* (Verissimo et al., 2014; IUCN, 2017), thus highlighting the importance of genetic work in identifying the Mediterranean species and better understand their biology and phylogeny.

2. Materials and methods

In the current study, the latest recognised taxonomic names are being used thus we are recognising the names *B. centroura* and *C. uyato* as the valid named for what were formerly known as *D. centroura* and *C. granulosus* in the Mediterranean Sea.

2.1. Specimen collection and morphological identification

All tissue samples obtained in this study were collected from dead specimens caught by Maltese fishermen and landed at the Malta fish market between 2003 and 2016, while a specimen was obtained from a stranding in 2017. Therefore, no specimen was sacrificed for this conservation study. Most of the specimens were caught as by-catch of other more economically important species. A total of 256 specimens, representing 24 species of sharks (genera: *Alopias*, *Carcharinus*, *Carcharadon*, *Centrophorus*, *Dalatias*, *Galeus*, *Hexanchus*, *Heptranchias*, *Isurus*, *Mustelus*, *Odontaspis*, *Oxyrinchus*, *Prionace*, *Scyliorhinus*, *Somniosus*, *Squalus* and *Squatina*) and 12 species of rays (genera: *Bathytoshia*, *Dasyatis*, *Dipturus*, *Leucoraja*, *Myliobatis*, *Raja*, *Rostroraja* and *Torpedo*) were sampled to genetically distinguish the various species of elasmobranchs landed (Supplementary data - Table 1). Each specimen was first identified down to the species level using morphological identification keys (Compagno, 1984a, 1984b; Serena, 2005; Bariche, 2012; Froese and Pauly, 2016), with the exception of two shark specimens that had a number of missing body parts. In the case of *R. clavata* the specimens encountered exhibited polychromatism in colour patterns on their dorsal surface (Mnasri et al., 2009; Schembri, 2013; Vella et al., 2016). Specimens collected were classified into the various forms according to Mnasri et al. (2009). The collection analysed in this study included the uniform (n = 1), speckled (n = 3), spotted (n = 6), ocellated (n = 3), reticulated (n = 6) and marbled (n = 6) forms (Fig. 1). Similarly, multiple specimens of both *R. radula* and *Dipturus oxyrinchus* were collected as different specimens exhibited variations in colouration and presence of spots on the dorsal surface (Fig. 1).

2.2. DNA isolation

Small tissue biopsies were collected and were either frozen at − 20 °C or stored in 20% dimethyl sulfoxide solution saturated with sodium chloride or in 100% ethanol at 4 °C. The genomic DNA was extracted from 15 mg of tissue using proteinase K, phenol-chloroform extraction following Milligan (1998). The final DNA extracts were stored in TE buffer at − 20 °C.

2.3. PCR amplification and sequencing

The genetic analyses focused on two partial mitochondrial DNA protein coding sequences, COI and ND2 genes. COI was amplified using four primer combinations and amplification protocols as described in Ward et al. (2005), while the ND2 gene was amplified using two newly designed primers ND2-MetF (5′AAGCTYTTGGGCCCATACC) and ND2-TrpR (5′AGCTTTGAAGGCTTTTGGTYT). The amplification protocol of the latter gene was carried out in 25 μL reaction volume using ~50 ng DNA template, 1 × FIREPol® Master Mix [1.5 mM Mg^{2+}; 200 μM each dNTP; FIREPol® DNA polymerase] (Solis BioDyne, Estonia), and 0.5 μM of each primer. The PCR reactions were subject to an initial denaturation of 95 °C for 5 min; followed by 28 cycles of 95 °C for 45 s, 54 °C for 45 s, 72 °C for 1 min; and a final extension 72 °C for 15 min. For the Family Dasyatidae the protocol was modified by using a T_a of 57 °C for 15 s. Each PCR product was sequenced through 3730XL Genetic Analyzer (Life Technologies) using the respective forward and reverse primers.

2.4. Data analyses

Sequences were trimmed and the complimentary sequences of each individual were assembled using Geneious R10 (Kearse et al., 2012). All sequences were manually checked for consistencies. Sequences were then aligned using both the nucleotide alignment method and the transitional alignment method for protein alignment of ClustalW (Thompson et al., 1994) within Geneious R10 (Kearse et al., 2012). The final sequences were deposited in GenBank under accession numbers KY909334 - KY909843; MF405097 - MF405098 (Supplementary data - Table 2).

The ND2 and COI sequences were submitted to GenBank via Blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi), while COI were also submitted to the BOLD Species Level Barcode Records Identification Engine (http://www.boldsystems.org) to identify in which Barcode Index Number (BIN) each specimen belongs. The Tree Based Identification within BOLD was also used for hierarchical placement of the specimens analysed vis-à-vis the already available data. This allowed morphological identification to be confirmed by genetic identification through identity matches with designated sequence clusters.

After conducting the partition-homogeneity test available in PAUP* 4.0a152 (Swofford, 2002) the COI and the ND2 data of each specimen were concatenated to produce a 1600 bp sequence. The haplotype diversity and nucleotide diversity within the species was conducted via Arlequin v3 (Excoffier and Lischer, 2010). The interspecific and intraspecific p-distance has been measured using MEGA v7 (Kumar et al., 2016), as suggested by Srivathsan and Meier (2012) and Collins et al. (2012). Tree-based phylogenetic analyses using neighbour-joining (NJ) and maximum-likelihood (ML) was carried out for shark species and for rays separately. The NJ tree was constructed using p-distance (Collins et al., 2012; Srivathsan and Meier, 2012; Collins and Cruickshank, 2013), while the ML tree was constructed using GTR + G + I nucleotide substitution model, as the model of best fit for both shark and rays, identified through MEGA v7 (Kumar et al., 2016). Phylogenetic trees were constructed via MEGA v7 (Kumar et al., 2016) using 1000 bootstraps to attest the robustness of the trees. For some species, a parsimony haplotype network via TCS (Clement et al., 2000) was constructed using PopART (Leigh and Bryant, 2015) to analyse the association between the various haplotypes identified during this study.

3. Results and discussion

3.1. General

A total of 256 elasmobranch individuals, representing 36 species, were analysed using a 610 bp and a 990 bp sequence from COI and ND2 respectively. The nucleotide frequencies of the COI data were