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## Puzzling over spurdogs: molecular taxonomy assessment of the *Squalus* species in the Strait of Sicily

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### Abstract

The actual occurrence of *Squalus megalops* in the Mediterranean Sea has recently been questioned. Several research works which sought to assess available morphological and meristic features that differentiate *S. megalops* from other *Squalus* species in the Mediterranean Sea, revealed poor discriminatory power and high variability of the assessed characters, especially when comparing *S. megalops* and *S. blainville*. The application of molecular tools does not support the presence of *S. megalops*.

In the present study, we screened spurdog species from the Strait of Sicily using a molecular taxonomy approach based on two mitochondrial DNA markers and we report the occurrence of two *Squalus* lineages characterizing specimens collected from the stretch of sea between Tunisia, southern Sicily, Malta and Libya. The results support the hypothesis that a common species, *S. blainville*, currently inhabits the Mediterranean Sea, while a second and rare species is probably an occasional visitor with high morphological similarity to the *S. megalops* and *S. blainville* but is genetically distinct from both. Within this perspective, the occurrence of *S. megalops* in the Mediterranean Sea is not confirmed and our study highlights the taxonomic uncertainties in relation to the occurrence and distribution of *Squalus* species in this region. We encourage the establishment of a coordinated international effort to implement a comprehensive and integrated taxonomic assessment on this genus which represents an irreplaceable component of the biodiversity of the area.

**Keywords:** Mediterranean Sea, conservation, cryptic species, mitochondrial DNA, shark misidentification

### Introduction

The intrinsic low variation of morphological characters specific to elasmobranchs hinders their taxonomic identification at the species level and consequently undermines their conservation at different geographical scales (McEachran & Dunn 1998; Quattro et al. 2006; Aschliman et al. 2012). The lack of well-preserved holotypes for many shark species (e.g. *Centrophorus* spp.), misidentifications in databases and in the literature, and challenges in retrieving representative series of specimens for comparison are top-down impediments to the

proper taxonomic identification and the potential revision of genera (Veríssimo et al. 2014).

In particular, the genus *Squalus* Linnaeus, 1758 is distributed worldwide (Ebert & Stehmann 2013) and includes about 26 different species of long-lived sharks (Viana et al. 2016) inhabiting the waters of the continental shelf and upper slope, between 300–700 m of depth (Whitehead et al. 1984; Cannizzaro et al. 1995; Serena et al. 2009), as well as some seamounts and the waters around oceanic islands (Compagno 1984; Veríssimo et al. 2017). The genus is highly represented in bycatch and sev-

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eral studies have focused on the mitigation of the fishery impact on this group (Mandelman & Farrington 2007; Stoner & Kaimmer 2008; Tallack & Mandelman 2009). In addition, most spurdog shark species are included in the IUCN *Red List of Threatened Species* and are currently classified in categories from Data Deficient to Endangered (i.e. the Greeneye spurdog *S. chloroculus* Last et al. 2007; IUCN 2019). For this reason, highlighting the need to increase our knowledge about taxonomy, biology and ecology of these species appears now essential. Difficulties due to the problems of distinguishing between morphologically similar species and to the lack of effective and user-friendly identification field guides have been commented upon by many authors (Garrick 1960; Compagno 1984; Munoz-Chapuli & Ramos 1989; Verissimo et al. 2017). With the progressive expansion of molecular taxonomy (i.e. DNA barcoding) and its integration with morphological approaches, unravelling the taxonomy of confounding groups of sharks has become a priority for their conservation (Ebert et al. 2010; Verissimo et al. 2014; Pflieger et al. 2018). In more recent times, the well-established DNA barcoding technique based on the mitochondrial DNA (mtDNA) cytochrome *c* oxidase subunit I (COI) has been coupled with analysis of faster-evolving mitochondrial genes or long ribosomal DNA to improve the resolution and support of inferred phylogenetic relationship, up to the description of the evolutionary history of species (Avisé 2004; Naylor et al. 2012; Krehenwinkel et al. 2019).

Recently, Verissimo et al. (2017) reported uncertainties in the identification of *Squalus* specimens caught along the eastern Atlantic Ocean and in the Mediterranean Sea. In the latter area, *Squalus acanthias* Linnaeus, 1758 and *Squalus blainville* (Risso, 1827) are considered as resident species, while the presence of *S. megalops* (MacLeay, 1881) has been reported from Tunisia (Marouani et al. 2012). From the latter region, several specimens were analysed with a multidisciplinary approach and significant differences were reported for the two identified groups defined respectively as *S. blainville* and *S. megalops* (Marouani et al. 2012). Sequence data from both identified species were included later in the comprehensive assessment of the *Squalus* genus carried out in Verissimo et al. (2017). Using two mtDNA markers, three main groups and four main Clades corresponding to *S. acanthias* (Clade A), *S. blainville*/*S. megalops* (Clade B), *S. megalops* (Clade C) and *Squalus mitsukurii* Jordan & Snyder, 1903 (Clade D) were identified in the Eastern Atlantic and the Mediterranean Sea (Verissimo

et al. 2017). Individuals classified under Clade C, which is highly divergent from both *S. megalops* from Australian waters and Clade B, originates from tropical West African coasts, with the exception of the single specimen from Tunisia, previously identified as *S. blainville* by Marouani et al. (2012). The sequence data associated with the individual from Tunisian waters described as *S. megalops* in Marouani et al. (2012) fitted in Clade B. Given the above described results and since specimens classified as potential *S. megalops* are included in both Clade B and Clade C, these findings further support current inconsistencies in species identification within the genus *Squalus* and the need for an accurate redescription of *Squalus* species, especially in the Mediterranean Sea, to stabilize the systematic and facilitate specimens identification.

Extensive studies conducted in other areas of the Mediterranean Sea considering both morphological (chondrocranium and other body traits) and genetic (COI sequences) analyses, revealed the presence of only one spurdog species, *S. blainville*, in the Ionian, Lybian, Aegean Seas and in the Sardinian waters (Kousteni et al. 2016; Bellodi et al. 2018). These findings spotlighted the stretch of sea between Tunisia, southern Sicily, Malta and Libya, known as the Strait of Sicily, as a more interesting area for spurdog species. Differently from Marouani et al. (2012), Bonello et al. (2016) did not identify diagnostic features (e.g. dermal denticles) which could effectively discriminate between *S. blainville* and *S. megalops*. The presence of *S. blainville* in the Maltese waters was assessed through the use of the DNA barcoding approach (Bonello et al. 2016). In the same region, Vella et al. (2017) collected and analysed individuals belonging to the nominal *S. blainville* and genetically clustering within Clade B (*sensu* Verissimo et al. 2017), while three individuals were classified as *Squalus* sp. by the authors as clustering in the genetic Clade C (*sensu* Verissimo et al. 2017).

In the present study, we screened all spurdogs caught in the Strait of Sicily between 2016 and 2017 to investigate the species composition in this stretch of sea. Comparing new and publicly available mtDNA gene sequences (COI and NADH dehydrogenase subunit 2; NADH2) we investigated the possible co-occurrence of two or more species of spurdog within the study area. Furthermore, since previous studies revealed that nominal species characterized by a wide geographical distribution share mitochondrial lineages, we evaluated the relationship between the genetic lineages retrieved in the Strait of Sicily and other Clades previously identified within the genus *Squalus*.

## Materials and methods

### Sample collection and DNA extraction

A total of 160 individuals of *Squalus* were caught off the southern coast of Sicily (General Fisheries Commission for the Mediterranean Geographical Subarea, GFCM-GSA 16) between July 2016 and May 2017 at depths between 83 m and 452 m, in the framework of the International Bottom Trawl Survey in the Mediterranean programme (MEDITS, Spedicato et al. 2019) and the Campionamento Biologico (CAMP-BIOL) monitoring programme of commercial catch (Milisenda et al. 2017). Twelve additional samples of *Squalus blainville* caught around the Maltese Islands (GFCM-GSA 15) in 2012 (COI analyses included in Bonello et al. 2016) and ten specimens collected off the Tuscany coasts (GFCM-GSA 9) in 2017 were included in this study. All specimens were preserved at  $-20^{\circ}\text{C}$  until the collection of main biological data and tissues samples for genetic analyses. In particular, individual muscle tissue or fin clips were preserved in 96% ethanol for laboratory analyses (see Table S1 for details on the field species assignment and geographical origin of specimens).

Total genomic DNA (gDNA) was extracted from approximately 20 mg of tissue using the Wizard® SV Genomic DNA Purification System by Promega, according to the manufacturer's instructions. The quality of the extracted gDNA was assessed on a 0.8% agarose gel electrophoresis.

### mtDNA amplification and sequencing

A fragment of the mitochondrial gene COI (~650 bp) was obtained from each specimen by PCR using the FishF2 and FishR2 primers (Ward et al. 2005). PCR reactions were performed in 25  $\mu\text{l}$  total volume containing 2.5  $\mu\text{l}$  gDNA template, 10  $\mu\text{l}$  nuclease-free water, 5  $\mu\text{l}$  1x PCR buffer, 1.5  $\mu\text{l}$  of  $\text{MgCl}_2$  (50 mM), 2  $\mu\text{l}$  dNTPs (10 mM), 1.5  $\mu\text{l}$  of each primer (10  $\mu\text{M}$ ) and 0.25 U GoTaq G2 Flexi DNA polymerase (Promega). Amplifications were performed in a T-gradient thermocycler (Biometra) with an initial denaturation of 2 min at  $95^{\circ}\text{C}$ , followed by 35 cycles of 30 s at  $94^{\circ}\text{C}$ , 30 s at  $54^{\circ}\text{C}$  and 60 s at  $72^{\circ}\text{C}$  and a final extension step for 7 min at  $72^{\circ}\text{C}$ .

Similarly, a fragment of the NADH2 (~1100 bp) gene was amplified for all individuals using the Met-F and Trp-R primers (Vella et al. 2017). PCR conditions consisted of an initial denaturation of 2 min at  $95^{\circ}\text{C}$ , followed by 28 cycles of 45 s at  $95^{\circ}\text{C}$ , 45 s at  $54^{\circ}\text{C}$  and 60 s at  $72^{\circ}\text{C}$  and a final extension step for 7 min at  $72^{\circ}\text{C}$ . PCR outcomes were evaluated on

a 2% agarose gel and preserved at  $4^{\circ}\text{C}$  until purification. All PCR products were purified using the ExoSAP-IT™ Express PCR Product Cleanup Reagent (ThermoFisher Scientific) following the manufacturer's protocol. All amplicons were then sequenced with the same primers used for the amplification by the external provider MacroGen Europe (Amsterdam, the Netherlands).

### Data analysis

The mtDNA COI and NADH2 sequences electropherograms were imported in MEGA v.X (10.1; Kumar et al. 2018), and carefully assessed and edited. All the sequences for each marker were aligned with the CLUSTAL W algorithm (Thompson et al. 1994) as implemented in MEGA and the correct amino acidic translation was verified to exclude nuclear mitochondrial pseudogenes (Song et al. 2008).

The software DnaSP v.6 (Rozas et al. 2017) has been used to compute the number of haplotypes, the number of polymorphic and parsimony-informative sites, the haplotype (Hd) and nucleotide diversity (Pi) were estimated for each of the newly obtained COI and NADH2 datasets.

Available COI and NADH2 sequences were retrieved for *S. acanthias*, *Squalus albifrons* Last et al. 2007, *S. blainville*, *Squalus brevirostris* Tanaka, 1917, *Squalus chloroculus* Last et al. 2007, *Squalus clarkae* Pflieger et al. 2018, *Squalus crassispinus* Last, Edmunds & Yearsley, 2007, *Squalus cubensis* Howell Rivero, 1936, *Squalus edmundsi* White, Last & Stevens, 2007, *Squalus formosus* White and Iglesias (2011), *Squalus grahami* White, Last & Stevens, 2007, *Squalus griffini* Phyllipps, 1931, *Squalus hemipinnis* White, Last & Yearsley, 2007, *Squalus japonicus* Ishikawa, 1908, *S. megalops*, *S. mitsukurii*, *Squalus montalbani* Whiteley, 1931, *Squalus nasutus* Last, Marshall & White, 2007, *Squalus raoulensis* Duffy & Last, 2007 and *Squalus suckleyi* (Girard, 1855) from both BOLDsystems v.4 (Ratnasingham & Hebert 2007; <http://www.boldsystems.org>) and NCBI databases (<https://www.ncbi.nlm.nih.gov>) and added to the original dataset (see Table S2). In both datasets, when possible, retrieved data had different geographic origin to properly assess intraspecific variability.

For each mtDNA marker, a Neighbour-joining (NJ) tree (Saitou & Nei 1987) was computed with MEGA using p-distance (Collins & Cruickshank 2013) and, although no nucleotide gap was detected across both datasets, the 'pairwise deletion' option for the treatment of gaps and missing data was

selected. A bootstrap test (BS) with 10,000 replicates (Felsenstein 1985) was performed to evaluate the robustness of reconstructions. The average intra and interspecific genetic distances among the clades observed were calculated with MEGA.

In order to obtain more robust and comparable results in relation to previous studies (Marouani et al. 2012; Bonello et al. 2016; Vella et al. 2017; Verissimo et al. 2017), a concatenated dataset was created merging the newly obtained COI and NADH2 individual sequences with those published by Verissimo et al. (2017) and Vella et al. (2017). The two mtDNA genes were concatenated in a congruent dataset (see Table S2), using Phyutility v.2.2 (Smith & Dunn 2008). A second NJ tree was computed on the concatenated dataset as reported above.

The software IQ-TREE v.1.6.12 (Nguyen et al. 2015) was firstly used to identify the best substitution model to be applied to the concatenated dataset and then to compute the Maximum-Likelihood (ML) reconstruction using the TN+F+ G4 model and an ultrafast BS (Hoang et al. 2018) with 10,000 replicates.

The Bayesian Inference (BI) reconstruction was unravelled with MrBayes v.3.2.7 (Ronquist et al. 2012) using two independent runs of 1,000,000 generations and a 25% burn-in cut-off. Run convergence was assessed considering a mean standard deviation of split frequencies of <0.01 between runs.

For all the topology reconstruction methods, *Cirrhigaleus asper* (Merrett, 1973) (COI and NADH2 Accession numbers MN982926 and JQ518974, respectively) and *Cirrhigaleus australis* White, Last & Stevens, 2007 (NC\_024059) were chosen as outgroups for the analyses. The acquired trees were summarised in one congruent topology and edited using FigTree v.1.4.2 (Rambaut & Drummond 2012). BS values for NJ and ML trees were reported near nodes, as well as the posterior probability ( $P$ ) obtained for the BI reconstruction.

The phylogeographic relationship of species haplotypes was inferred with the Median Joining Tree clustering algorithm (Bandelt et al. 1999) implemented in the software PopART (Leigh & Bryant 2015). The network was obtained considering all the *Squalus* spp. nominal species and lineages detected across the concatenated dataset, represented by *S. acanthias*, *S. blainville*, *S. griffini*, *Squalus* cf. *megalops* (Mauritius), *Squalus* cf. *mitsukurii* (Uruguay and Hawaii), *Squalus* sp. Clade B (*sensu* Verissimo et al. 2017), *Squalus* sp. Clade C (*sensu* Verissimo et al. 2017) and *Squalus* sp. Clade D (*sensu* Verissimo et al. 2017).

## Results

DNA was successfully extracted from all tissue samples, although PCR amplification and sequencing were successful for 121 and 107 individuals for COI and NADH2, respectively. These newly obtained sequences of *Squalus* specimens collected in the Strait of Sicily and Tuscany coasts, have been deposited in GenBank under the Accession Numbers (COI Accession Numbers MW537886-MW537998; NADH2 Accession Numbers MW557187-MW557293).

The new COI sequences counted a total of 51 polymorphic and 33 parsimony informative sites, 54 mutations and 33 haplotypes ( $Hd = 0.781 \pm 0.034$ ;  $Pi = 0.005 \pm 0.027$ ), while a total of 54 polymorphic and 33 parsimony informative sites, 56 mutations and 39 haplotypes ( $Hd = 0.874 \pm 0.022$ ;  $Pi = 0.006 \pm 0.022$ ) were detected among new NADH2 sequences.

After retrieving additional sequences from public data repositories, the final dataset for COI included 735 sequences (499 bp long) representing 18 nominal species, while the NADH2 dataset included 352 sequences (523 bp long) representing 20 nominal species. The concatenated dataset consisted of 222 sequences (1022 bp long).

Considering the congruence among the NJ, ML and BI tree topologies obtained using the concatenated dataset (Table S2), all reconstructions were summarised in one topology (Figure 1), which assigned most of the individuals collected off the southern coast of Sicily, Malta and Tuscany to a bigger cluster including *S. blainville* from the Mediterranean Sea and *Squalus* sp. Clade B (*sensu* Verissimo et al. 2017) from South Africa, Angola, Namibia, Morocco, Portugal and the Mediterranean Sea (BS = 100% for both NJ and ML and posterior probability,  $P = 1$  for BI). Conversely, two specimens of *Squalus* (S7 and S9) are included within the bigger group composed by individuals of *Squalus* sp. collected in Gabon and Guinea, representing the genetic Clade C (*sensu* Verissimo et al. 2017; BS = 100% for both NJ and ML and  $P = 1$  for BI). Furthermore, the three *Squalus* sp. collected around Malta by Vella et al. (2017) were grouped within Clade C (Figure 1).

The NJ tree topologies reconstructed considering a large number of nominal species characterised by a wide geographic distribution (e.g. Australia, China, Indonesia, New Zealand, Japan, Taiwan, UK, USA) have consistently highlighted the existence of three distinct and well-supported groups (Figure S1, for COI; Figure S2, for NADH2). A first group (group I; Table S2)

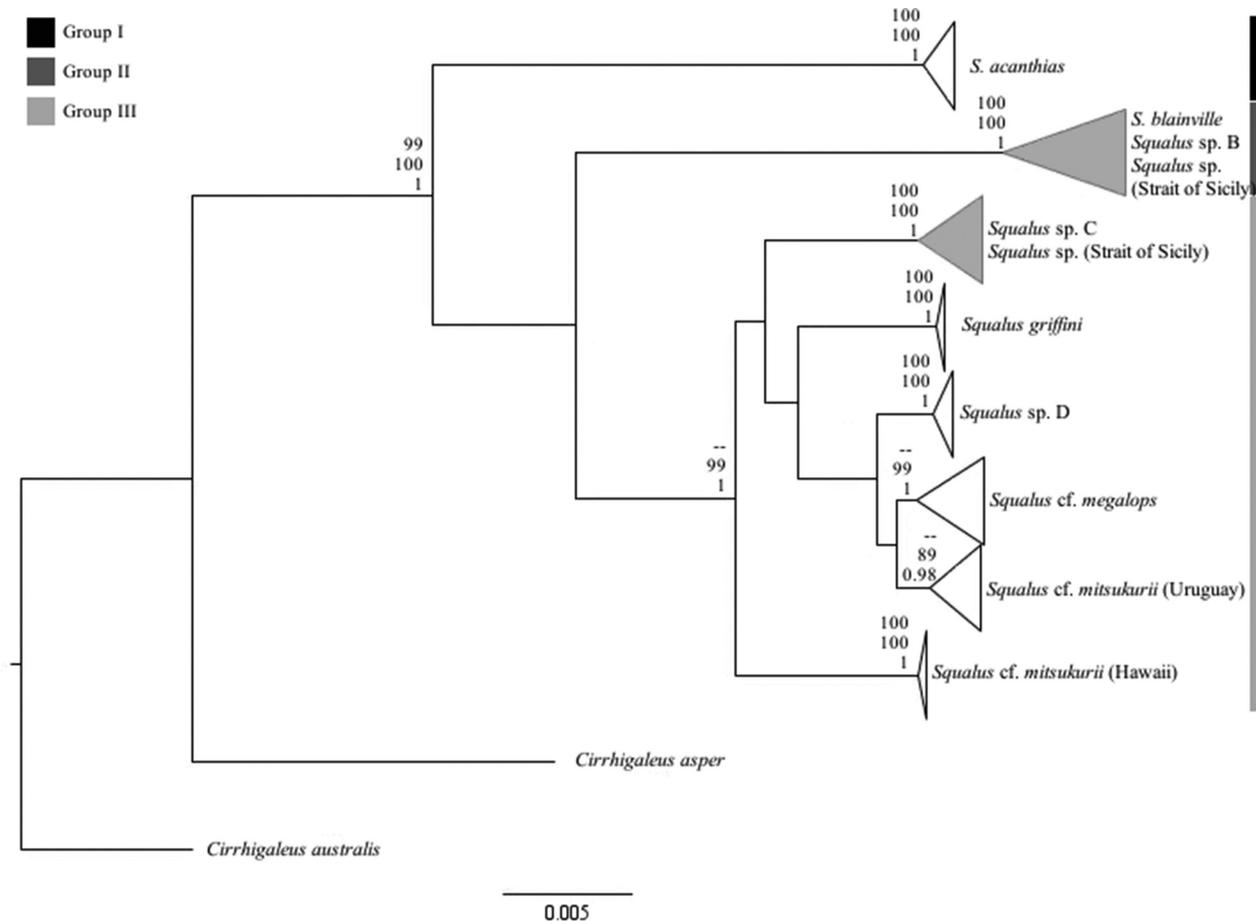


Figure 1. Tree topology based on concatenated mitochondrial sequences (COI and NADH2) summarising NJ, ML and BI reconstructions of the genus *Squalus*. BS values of NJ and ML and Bayesian posterior probability  $P$  are respectively reported top-down near nodes. BS values are reported when  $\geq 70\%$ .  $P$  is reported when  $\geq 0.95$ . Non-supported nodes are indicated as a double dash. Clades including samples of *Squalus* collected in the Strait of Sicily are highlighted in grey.

included the two main Clades of *S. acanthias* and *S. suckleyi* with high BS values in both COI and NADH2 reconstructions (100% and 99%, respectively), a second one (group II; Table S2) included the nominal *S. blainville*, *S. brevirostris*, *S. megalops* and *S. raoulensis* (BS = 100% in COI tree topology and BS = 99% in NADH2), and a last one (group III in Table S2) included all the other species considered with BS = 99% in COI.

Considering the COI mitochondrial gene, the genetic distance within groups was generally low, ranging from 0% (*Squalus albifrons*, *S. grahami*, *S. griffini*, *Squalus* cf. *megalops*, *Squalus* cf. *mitsukurii* from Hawaii) to 1.47% (*S. nasutus*) and 1.49% (*S. megalops*; Table S3). This was also confirmed by the NADH2 data, for which the genetic distance measured in *S. mitsukurii* complex sp. D and *S. raoulensis* was also 0%, while in *S. brevirostris* the genetic distance reached 3.02% (Table S4).

COI genetic distances between groups ranged from 0.10% (*S. clarkae* vs *S. cubensis*) to 7.90% (*S. acanthias* vs *S. megalops* or *S. acanthias* vs *S. raoulensis*; Table S3, for COI), while they ranged from 0% (*S. mitsukurii* complex sp. D vs *Squalus* sp. Clade D) and 8.78% (*S. edmundsi* vs *S. suckleyi*) for NADH2 (Table S4). COI genetic distances increased when comparing *Squalus* individuals caught in the Strait of Sicily with *S. megalops* from Australia (1.40%), with *Squalus* cf. *megalops* from Mauritius (6.50%) and with *Squalus* sp. Clade C (*sensu* Verissimo et al. 2017), which included individuals S7 and S9 collected in the Strait (6.60%; Table S3). NADH2 genetic distances showed similar results. In particular, distances increased when comparing *Squalus* sp. caught in the Strait of Sicily with *S. blainville* (0.52%), with *Squalus* sp. Clade B (*sensu* Verissimo et al. 2017; 0.56%), with *Squalus* cf.

*megalops* from Mauritius (4.95%) and with *Squalus* sp. Clade C (*sensu* Verissimo et al. 2017), which included S7 and S9 individuals (5%; Table S4).

The haplotype network in Figure S3 showed the presence of two most frequent haplotypes shared by almost all the samples ascribable to *S. blainville*. Clusters of fewer individuals sharing the same haplotype, down to one individual displaying only a different variant, are separated from one to five mutational steps. The *S. blainville* haplogroups were separated from *Squalus* samples collected in Sicily and Malta and shared haplotypes with *Squalus* sp. Clade C from Gabon and Guinea, by at least 68 mutations. Furthermore, 59 mutational steps separated the *S. blainville* haplogroups from *Squalus* cf. *megalops* (Mauritius), while the latter was separated from *Squalus* sp. Clade C by 24 mutations.

## Discussion

The use of molecular taxonomy is largely considered a powerful tool for the correct assessment of specimens identification, the discovery of new species and, in some cases, also the identification of cryptic (Duncan et al. 2006; Corrigan et al. 2008; Liu et al. 2013) and/or intricate complexes of species (Ward et al. 2008; Arlyza et al. 2013). Indeed, among sharks and batoids, new species have been described (De Astarloa et al. 2008; Smith et al. 2008; White & Iglesias 2011; Daly-Engel et al. 2018; Pflieger et al. 2018), old species resurrected (Ebert et al. 2010; Viana & de Carvalho 2018), and identification issues have been resolved with DNA barcoding when the morphological methods gave misleading results (Bonello et al. 2016; Cariani et al. 2017).

The lack of robust data from original descriptions compromises the correct identification of specimens with implications for species conservation and management purposes. This is particularly true for many genera, *Squalus* included (Verissimo et al. 2017). The integration of morphological and molecular taxonomy techniques has been proved helpful (Henderson et al. 2016). In the last years, few studies in the context of integrative taxonomy were successfully performed on the genus *Squalus* aiming at the integration of new molecular taxonomy techniques to more classical morphological analyses with the aim to clarify taxonomic ambiguities between some of the species (Marouani et al. 2012; Bonello et al. 2016).

The evidence obtained in the present study, which followed in the footsteps of previous case studies in terms of genetic methodologies and analytical approach (Ward et al. 2007; Naylor et al. 2012;

Vella et al. 2017; Verissimo et al. 2017; Bellodi et al. 2018), confirmed the branching of the genus *Squalus* into three main lineages as referring to *Squalus acanthias*/*S. suckleyi* (group I), *S. blainville*/*S. megalops*/*S. raoulensis*/*S. brevirostris* (group II) and a heterogeneous group of species mainly represented by the *S. mitsukurii* species complex (group III). At a finer resolution, the assignment of public data of *Squalus* individuals to Clades A-D (*sensu* Verissimo et al. 2017) was confirmed by robust tree topologies. Almost all the individuals sampled in the Strait of Sicily and classified as *Squalus* sp. clustered in Clade B (*sensu* Verissimo et al. 2017) and thus we suggest that they could be assigned to the nominal species *S. blainville*. On the other hand, two individuals sampled in the Strait of Sicily and classified as *Squalus* sp. fell in Clade C, together with four more specimens collected in the adjacent Tunisia (BOLD: FOAI329-09) and Malta waters (data from Vella et al. 2017) and showed a genetic distance from the former Clade B as high as the one existing between well-distinguished nominal species (e.g. *S. mitsukurii* and *S. raoulensis* from group III and II, respectively). Similar distances between genetic lineages were described in Verissimo et al. (2017).

Therefore, here we confirm the hypothesis that specimens of Clade C are not related to the Australian *S. megalops*, since these lineages are separated by a distance of 6.20%. In fact, individuals of *Squalus* sp. from Clade C are closer to *S. megalops* from Mauritius (2.30% according to the COI gene). This was confirmed by the species-haplotype network, where at least 68 mutational steps separated *Squalus* sp. clustering in Clade B (ascribable to *S. blainville*) from *Squalus* sp. clustering in Clade C from tropical Western Africa. Similar haplotype differentiation between these same lineages was observed by Vella et al. (2017), who reported 83 mutations across a concatenated dataset 1600 bp long.

Hence, here we support previous studies that suggest that *S. megalops* does not occur in the eastern Atlantic and Mediterranean waters and that individuals composing Clade C should be considered a new species that needs formal description and proper taxonomic assessment (Last & Stevens 2009; Verissimo et al. 2017). As a matter of fact, the occurrence of *S. megalops* appears more likely limited to the Australian waters, since the tree topologies obtained herein showed *Squalus blainville* from the Strait of Sicily (this study) clustering with long-nose spurdog *S. blainville* from Sardinian coasts (Bellodi et al. 2018), Malta (Bonello et al. 2016;

Vella et al. 2017), Tuscany (this study), Spain, Libyan and Ionian Seas, Greece (Kousteni et al. 2016) along with *Squalus* sp. Clade B (Verissimo et al. 2017). Unfortunately, the sequence data from Marouani et al. (2012) are not publicly available and thus a direct comparison was not possible.

The results discussed here, reinforced by both the genetic distances measured between groups and clades and the haplotype network, support the hypothesis that a common species, *S. blainville*, is currently inhabiting the Mediterranean Sea, while a second and extremely rare one, which has not yet been extensively described, is probably an occasional visitor of the Strait. This second species shows a strikingly high morphological similarity to *S. blainville*, but it is genetically distinct from both *S. blainville* and *S. megalops*. Within this perspective, the absence of records of *S. megalops* in the Atlantic Ocean and Mediterranean Sea (Straube et al. 2013; Verissimo et al. 2017; Viana & de Carvalho 2018) can be confirmed. As previously mentioned, cryptic speciation among elasmobranchs is very common (Borsa et al. 2016, 2018) and the number of new descriptions, re-descriptions and resurrections of species is growing with the increasing application of molecular tools and integrated taxonomic methodologies. Starting from the accurate morphological data registered in Marouani et al. (2012) a dedicated effort is needed to identify and assess diagnostic features that characterize the individuals ascribed to Clade C exhibiting high morphological similarity with both species (*S. megalops* and *S. blainville*). What is more, a comparative approach involving both mtDNA markers and highly polymorphic nuclear DNA loci, besides morphology, is recommended to enhance the power of analyses aiming at unravelling the gene flow and migratory patterns of such a rare and geographically limited species.

To conclude, current inconsistencies in species identification within the genus *Squalus* need to be resolved and an accurate redescription of *Squalus* species is advised, especially in the Mediterranean where, along with commercial fishery, the bycatch of sharks may lead to the erosion of local biodiversity including genetic diversity. For all these reasons, the growing concern about the vulnerability of sharks to fishing pressure (Dulvy et al. 2014) and overexploitation (Simpfendorfer & Kyne 2009) now requires strong measures for species protection and management, especially in this exploited stretch of Sea.

To continue the acquisition of new information and the resolution of old problems, the establishment and strengthening of an international network

of collaborations and the maximisation of sampling effort would go a long way towards filling the gaps in our knowledge of these shark species, which represent an irreplaceable component of biodiversity, in terms of both species and genetic richness, to be protected and conserved before they are “Lost Before Found” (White et al. 2019).

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No potential conflict of interest was reported by the authors.

### Supplementary material

Supplemental data for this article can be accessed [here](#).

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