Molecular and morphological evidence of the occurrence of the Norwegian skate *Dipturus nidarosiensis* (Storm, 1881) in the Mediterranean Sea

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**Abstract**

Fourteen specimens of the Norwegian skate, *Dipturus nidarosiensis* (Rajiformes, Rajidae), were caught off the Sardinian coasts (Central Western Mediterranean Sea) in 2005–2008 between 600 and 1420 m of depth. Their identification has been confirmed by the sequencing of three regions of the mtDNA (16SrDNA, control region and cytochrome c oxidase subunit 1) and comparison of the obtained sequences with that of three species of *Dipturus* (*D. batis*, *D. oxyrinchus* and *D. nidarosiensis*) from the Mediterranean Sea and the adjacent North-eastern Atlantic Ocean. A simple PCR-RFLP assay has been developed for an easy, reliable and robust identification of these skate species. A morphological comparison of the Norwegian skate with congeneric species is given in order to help future identifications. This is the first record of *D. nidarosiensis* in the Mediterranean Sea; the possibility of recent or ancient but unnoticed occurrence of the Norwegian skate in the region is discussed.

**Key words:** 16SrDNA, COI, control region, *Dipturus nidarosiensis*, Mediterranean Sea

**Introduction**

Skates (order Rajiformes, family Rajidae) are an extremely diverse group of fishes, characterized by a high morphological conservatism (McEachran & Dunn 1998). In recent years, the number of nominal species has increased exponentially, with more species described in the last 60 years than in the previous 200 years and another 50–100 species still to be described (Ebert & Compagno 2007).

*Dipturus* (Rafinesque, 1810) is the second most speciose genus within Rajidae, with most of its species described in the last 40 years. Five species of *Dipturus* and an undescribed species are known from the eastern Atlantic, and two of these also occur in the Mediterranean Sea. In the eastern Atlantic region, *Dipturus batis* (Linnaeus, 1758), *Dipturus oxyrinchus* (Linnaeus, 1758), *Dipturus linteus* Fries, 1839, *Dipturus dourei* (Cadenat, 1960) and *Dipturus nidarosiensis* (Storm, 1881) are present, together with a further species (*Dipturus* sp.) which has yet to be formally described (Gibson et al. 2008). Two of these species, *D. batis* and *D. oxyrinchus*, occur in the Mediterranean Sea (Serena 2005).

According to the List Categories and Criteria of the IUCN, a recognized objective system for assessing the global risk of extinction for species (Vié et al. 2008), three out of five described *Dipturus* are considered threatened. The Norwegian skate *Dipturus nidarosiensis* is listed as near threatened (Stehmann 2008). It is known as the only endemic species of *Dipturus* to the NE Atlantic area. Despite its common name, *D. nidarosiensis* is a very rare species even in the Norwegian area where today it is very rarely caught (Williams et al. 2008). Like other large skates, *D. nidarosiensis* has a low reproductive rate and is vulnerable to trawl fisheries; therefore, this species needs careful monitoring.
Unfortunately, catch and landing data for skates are often of poor quality because of a general lack of species-specific recording (Johnston et al. 2005). Skates are difficult to identify, misidentifications can be quite common (Daan 2001), and this is especially true for the NE Atlantic *Dipturus* species. Actually, except for the more visually distinct species such as *D. nidarosiensis*, there is still concern regarding the validity of the skate identifications in the entire NE Atlantic area (Williams et al. 2008). This taxonomic impediment can hamper the assessment, conservation and management of global fish biodiversity (Ward et al. 2009).

On deep trawl surveys and commercial fishing operations off Sardinia (Central Western Mediterranean Sea) in 2005–2008, 14 *Dipturus* specimens were collected between 600 and 1420 m. As their external features did not match with the description of the two species known for the Mediterranean Sea, the molecular genetic technique of DNA barcoding was utilized for their identification.

To facilitate the identification of the *Dipturus* skates found in the Mediterranean Sea, a rapid PCR-RFLP assay was developed on an amplified segment of the mitochondrial DNA Control Region.

Morphological comparisons with other species of *Dipturus* were performed to provide useful data in future identifications.

**Material and methods**

**Samples**

Fourteen specimens of *Dipturus* sp. were collected in the Sardinian Channel (Figure 1) from 2005 to 2008: 9 adult females, 1 adult male, 2 sub-adult males, and 2 juvenile males. Twelve of these individuals were caught during experimental deep bottom trawl surveys carried out by the DBAE (Dipartimento di Biologia Animale ed Ecologia, University of Cagliari) from 800 to 1420 m depth, and two further specimens were caught in the same area by commercial trawl fishermen at 600 m depth and delivered to the DBAE laboratory. All captures were made in the area delineated by the coordinates N 38°58'38.48 – E 09°46'09.20. After capture, the specimens were frozen on board, transferred to the laboratory, measured, and photographed dorsally and ventrally (Figure 1). Due to the very large size of the adult specimens, the whole body of only two adults (one adult male and one adult female) and of the two young specimens were preserved and kept for further inspections at the DBAE Zoological collection, Museum of Zoology, University of Cagliari (Table I).

Mediterranean specimens (*n* = 12) of long-nosed skate *D. oxyrinchus*, caught around Sardinia during 2005 and 2006 in Medits (Bertrand et al. 2002) and Grund (Relini 2000) experimental trawl surveys at depths from 130 to 660 m, were analysed. After the measurements, photography and tissue sampling, one female and one male of *D. oxyrinchus* were preserved and kept at the DBAE Zoological collection (Table I).

Other samples of *D. oxyrinchus*, *D. batis* and *D. nidarosiensis* specimens were collected in the NE Atlantic by S. Iglesias and colleagues; all but two of the Atlantic specimens are preserved in the Muséum National d’Histoire Naturelle of Paris (MNHN) (Table I).

**Morphology**

Measurements were recorded for all the Mediterranean specimens. Colour was recorded for fresh individuals. Some external measurements were made, as described by Hubbs & Ishiyama (1968) and Stehmann (1995) (Table II and Supplementary Tables S1 and S2). Supplementary tables are available on the supplementary content tab of this articles page on www.informaworld.com/mbr).

**Genetic analyses**

Muscle tissues from the underside of the pectoral fins were sampled from frozen specimens then stored in absolute ethanol at 4°C and kept in the genetic tissue repository at the University of Cagliari (DBAE) while the Atlantic tissue samples were stored at the ‘Station de Biologie Marine de Concarneau’ (BPS) (Table I).

Genomic DNA was extracted according to a salting-out method (Miller et al. 1988). All samples were analysed using polymerase chain reaction (PCR) and direct DNA sequencing. PCR was used to selectively amplify the 16S ribosomal mitochondrial gene (16SrDNA) using the primers described in Palumbi et al. (1991), the control region (CR) using the primers ElDloopF and RajinaeP7r (Valsecchi et al. 2005), the 3' end of the cytochrome oxidase subunit I (COI) with the primers RajaCOIf and RajaCOIr (Alvarado Bremer et al. 2005). Individual haplotypes were aligned with CLUSTAL-W implemented in MEGA version 4 (Tamura et al. 2007).

To estimate the genetic distances between pairs of DNA sequences we used the Kimura 2-parameter distance (DK2P; Kimura 1980) with the deletion of ambiguous or missing bases in pairwise sequence comparisons. Within- and among-clade distances were calculated and neighbor-joining (NJ) phylogenetic analyses were carried out in MEGA 4.0.
Support for nodes for NJ analyses was assessed by non-parametric bootstrapping (1000 replicates).

To facilitate the identification of the *Dipturus* skates found in the Mediterranean Sea, a rapid PCR-RFLP assay was developed based on the combined restriction activity of two endonucleases *DdeI* (Invitrogen) and *TaqI* (Invitrogen) on the amplified segment of the mitochondrial DNA Control Region.

Results

Morphology

The specimens of skate caught in Sardinia clearly belonged to the genus *Dipturus* Rafinesque 1810 in having the combination of the following characters: a long, hard rostral cartilage (length more than 60% of the dorsal head length), disc rhomboid and nearly free of denticles with few thorns (Compagno 1999),
Table I. Sampling site, collection numbers (for the Atlantic samples Station de Biologie Marine de Concarneau/Museu National d’Histoire Naturelle of Paris =BPS/MNHN, for the Mediterranean samples Università di Cagliari/Dip. Biologia animale ed ecologia =DBAE), haplotype code and relative GenBank Accession numbers of the 16SrDNA, CR and COI sequences.

| Species       | Sampling site | Coll. no.   | Size (mm) | Sex | 16S hap | GenBank no. | CR hap | GenBank no. | COI hap | GenBank no. |
|---------------|---------------|-------------|-----------|-----|---------|-------------|--------|-------------|---------|-------------|
| *Dipturus nidarosiensis* | NE Atlantic     | MNHN2004-0814 | 827       | M   | 16hap1  | EF081266*  | CRhap1 | EU915266    | COIhap1 | FJ347546    |
|                | NE Atlantic     | BPS-0547     | 1047      | M   | 16hap2  | EF647876   | CRhap1 | EU915266    | COIhap1 | FJ347544    |
|                | NE Atlantic     | MNHN2003-0541 | 317       | M   | 16hap2  | EF647876   | CRhap1 | EU915266    | COIhap1 | FJ347544    |
| Mediterranean  | DBAE-M05C11     | 1180         | F         | 16hap2| EF647876 | CRhap1     | EU915266| COIhap1     |        |             |
| Mediterranean  | DBAE-M05C12     | 980          | M         | 16hap2| EF647876 | CRhap1     | EU915266| COIhap1     |        |             |
| Mediterranean  | DBAE-R06C1      | 1482         | F         | 16hap2| EF647876 | CRhap1     | EU915266| COIhap1     |        |             |
| Mediterranean  | DBAE-1M06C07**  | 1180         | F         | 16hap2| EF647876 | CRhap1     | EU915266| COIhap1     |        |             |
| Mediterranean  | DBAE-R07C1      | 1220         | F         | 16hap2| EF647876 | CRhap1     | EU915266| COIhap1     |        |             |
| Mediterranean  | DBAE-M07C7**    | 240          | M         | 16hap2| EF647876 | CRhap1     | EU915266| COIhap1     |        |             |
| Mediterranean  | DBAE-M07C9      | 1000         | F         | 16hap2| EF647876 | CRhap1     | EU915266| COIhap1     |        |             |
| Mediterranean  | DBAE-R08C1      | 1200         | F         | 16hap2| EF647876 | CRhap1     | EU915266| COIhap1     |        |             |
| Mediterranean  | DBAE-M06C01**   | 242          | F         | 16hap2| EF647876 | CRhap2     | EU915267| COIhap1     |        |             |
| Mediterranean  | DBAE-M06C10     | 1320         | F         | 16hap2| EF647876 | CRhap2     | EU915267| COIhap1     |        |             |
| Mediterranean  | DBAE-1M06C14    | 1288         | F         | 16hap2| EF647876 | CRhap2     | EU915267| COIhap1     |        |             |
| Mediterranean  | DBAE-2M06C14    | 1058         | M         | 16hap2| EF647876 | CRhap2     | EU915267| COIhap1     |        |             |
| Mediterranean  | DBAE-2M06C07**  | 1180         | M         | 16hap2| EF647876 | CRhap3     | EU915268| COIhap2     |        |             |
| Mediterranean  | DBAE-M06C09     | 1365         | F         | 16hap2| EF647876 | CRhap4     | EU915269| COIhap2     |        |             |
| *Dipturus oxyrinchus* | NE Atlantic     | MNHN2004-1515 | 1295      | M   | 16hap3  | EF647874   | CRhap5 | EU915270    | COIhap4 | FJ347547    |
|                | NE Atlantic     | MNHN2004-1516 | 647       | M   | 16hap3  | EF647874   | CRhap5 | EU915270    | COIhap4 | FJ347547    |
| Mediterranean  | DBAE-2G05C41    | 923          | M         | 16hap3| EF647874 | CRhap5     | EU915270| COIhap4     |        |             |
| Mediterranean  | DBAE-8M05C01**  | 460          | F         | 16hap3| EF647874 | CRhap5     | EU915270| COIhap4     |        |             |
| Mediterranean  | DBAE-7M05C01    | 165          | M         | 16hap3| EF647874 | CRhap5     | EU915270| COIhap4     |        |             |
| Mediterranean  | DBAE-2G05C50    | 942          | M         | 16hap3| EF647874 | CRhap7     | EU915272| COIhap4     |        |             |
| Mediterranean  | DBAE-3M05C37    | 230          | M         | 16hap3| EF647874 | CRhap7     | EU915272| COIhap4     |        |             |
| Mediterranean  | DBAE-1M05C35    | 758          | M         | 16hap3| EF647874 | CRhap7     | EU915272| COIhap4     |        |             |
| Mediterranean  | DBAE-2M05C35    | 675          | M         | 16hap3| EF647874 | CRhap7     | EU915272| COIhap4     |        |             |
| Mediterranean  | DBAE-3M05C75**  | 920          | M         | 16hap3| EF647874 | CRhap8     | EU915273| COIhap5     |        |             |
| Mediterranean  | DBAE-1G05C21    | 858          | M         | 16hap3| EF647874 | CRhap9     | EU915274| COIhap4     |        |             |
| Mediterranean  | DBAE-1UM07C8    | /            | /         | 16hap3| EF647874 | CRhap9     | EU915274| COIhap4     |        |             |
| Mediterranean  | DBAE-1M05C90    | 804          | M         | 16hap3| EF647874 | CRhap9     | EU915274| COIhap4     |        |             |
| Mediterranean  | DBAE- AlgASAB1  | /            | /         | 16hap3| EF647875 | CRhap10    | EU915275| COIhap4     |        |             |
| *Dipturus batis* | NE Atlantic     | MNHN2003-0536 | 417       | F   | 16hap6  | EF081271*  | CRhap12 | EU915277    | COIhap7 | FJ347549    |
|                | NE Atlantic     | MNHN2004-1545 | 762       | M   | 16hap5  | EF081272*  | CRhap12 | EU915277    | COIhap7 | FJ347549    |
|                | NE Atlantic     | MNHN2004-1546 | 531       | F   | 16hap5  | EF081273*  | CRhap11 | EU915276    | COIhap7 | FJ347549    |
|                | NE Atlantic     | MNHN2004-1547 | /         | 16hap5| EF081274* | CRhap11    | EU915276| COIhap7     |        |             |
|                | NE Atlantic     | MNHN2004-1548 | /         | 16hap5| EF081275* | CRhap11    | EU915276| COIhap7     |        |             |

*From Iglesias et al. unpublished data (only nucleotide positions from 1979 to 2578 of the original sequence were included in the alignment); **specimens stored for future further inspections at the DBAE Zoological collection, Museum of Zoology, University of Cagliari.
anterolateral margin of disc concave, line connecting tip of snout to anterior aspect of lateral corner of disc not intersecting margin of disc after rhomboid disc (Stehmann & Burkel, 1984) and a total length greater than 55 cm when adult (Ishihara 1987). The Sardinian Dipturus specimens were distinct from both species of the genus thought to occur in the Mediterranean in colour pattern, pattern of tail thorns, snout length, number of tooth rows, total length, and depth occupied (Table II and Figure 2). However, the Sardinian specimens appeared similar or identical to D. nidarosiensis which is endemic to the NE Atlantic, apart from the reduced number of median thorns on the tail (presumably lost during fishing operations as suggested by the conspicuous scars) (Table II). In adults, both dorsal and ventral surfaces were uniformly dark (Figure 1a, b, e, f). The dorsal surface of juveniles was medium brown to grey/brownish, in some specimens darker; the ventral surface was dark brown, darker than the dorsal surface (Figure 1c, d). This is the main character that easily distinguishes D. nidarosiensis from the other species of Dipturus present in the Mediterranean/Eastern Atlantic; in the latter the underside is not uniformly coloured and often whitish or paler than the dorsal surface (Figure 2). Therefore, like D. nidarosiensis, the Sardinian specimens morphologically did not correspond to other eastern Atlantic Dipturus.

DNA sequence analyses

Haplotype codes and GenBank Accession numbers for the three genes analysed (16SrDNA, CR and COI) are listed in Table I. At least 600 bp of 16SrDNA nucleotide sequences were determined from Mediterranean D. oxyrinchus and Dipturus sp. individuals and compared with 16SrDNA sequences from Atlantic specimens of D. oxyrinchus, D. nidarosiensis and D. batis. In Atlantic D. oxyrinchus sequences, the variation in the 16SrDNA fragment defined two haplotypes (16Shap1 and 16Shap2), which differed from each other for one indel; all the 14 Mediterranean specimens of Dipturus sp. showed the same 16Shap2 haplotype which was differentiated by two transitions from the 16Shap4 haplotype of the D. oxyrinchus specimens. Therefore, like D. oxyrinchus, the Sardinian specimens were uniformly dark brown on both surfaces; underside uniformly dark brown with few black mucus pores (Table II). However, the Sardinian specimens appeared similar or identical to D. nidarosiensis which is endemic to the NE Atlantic, apart from the reduced number of median thorns on the tail (presumably lost during fishing operations as suggested by the conspicuous scars) (Table II). In adults, both dorsal and ventral surfaces were uniformly dark (Figure 1a, b, e, f). The dorsal surface of juveniles was medium brown to grey/brownish, in some specimens darker; the ventral surface was dark brown, darker than the dorsal surface (Figure 1c, d). This is the main character that easily distinguishes D. nidarosiensis from the other species of Dipturus present in the Mediterranean/Eastern Atlantic; in the latter the underside is not uniformly coloured and often whitish or paler than the dorsal surface (Figure 2). Therefore, like D. nidarosiensis, the Sardinian specimens morphologically did not correspond to other eastern Atlantic Dipturus.

Table II. Main morphological differences, useful for field identification of adults, among species of Dipturus occurring in Central, Northeast Atlantic and Mediterranean Sea.

| Species                | Colour pattern                                      | Medio-dorsal thorns on tail | Preorbital/interorbital width | Tooth rows (upper jaw) | Maximum size TL (cm) | Depth range (m) | Reference               |
|------------------------|-----------------------------------------------------|----------------------------|-------------------------------|-----------------------|----------------------|------------------|------------------------|
| Dipturus batis         | Upper surface olive-grey or brown with a variable pattern of light spots, underside ash-grey to blue-grey | 12-18                      | 2.5-4.0                      | 40-56                 | To about 250          | To about 600 (mainly within 200) | Stehmann & Burkel 1984 |
| Dipturus dourei        | Underside as dark as the upper surface              | from 11 to 20              | 4.5                          | 29-34                 | Up to 100             | Up to 1200       | Stehmann 1995          |
| Dipturus nidarosiensis| Plain dark on both surfaces; underside uniformly dark brown with few black mucus pores | 40-50                      | -                            | -                     | To > 200              | From about 200 to > 1000 | Stehmann & Burkel 1984 |
| Dipturus nidarosiensis (Sardinia) | Both sides grey-brownish               | 30-41                      | 3.2-4.4                      | 42-50                 | Up to 148 (females)   | From 600 to 1420 | Present paper          |
| Dipturus oxyrinchus    | Upper surface grey or brown, with spots and blots, underside grey | 4-11                       | 5.5-7.0                      | 30-40                 | To about 150          | From 90 to about 900 | Stehmann & Burkel 1984; Stehmann 1995 |
remaining individual. Both our *D. oxyrinchus*
16SrDNA haplotypes (*16Shap3* and *16Shap4*)
proved to be identical to the homologous portion
of the *D. oxyrinchus* haplotype by Tinti et al. (2003).
Finally, two 16SrDNA haplotypes (*16Shap5*
and *16Shap6*) were obtained for Atlantic *D. batis*.

For the COI gene 560 bp of nucleotide sequence
were determined from *D. oxyrinchus*, *Dipturus*
*sp.*, *D. nidarosiensis* and *D. batis* individuals, both of Medi-
terranean and Atlantic origin. Three different COI
haplotypes were found for *D. nidarosiensis*
(*COIhap1*/*C1*3), two different COI haplotypes were
found for *Dipturus* *sp.* (*COIhap1*/*C1*2), three COI
haplotypes for *D. oxyrinchus* specimens (*COIhap4,
5, 8*) and one COI haplotype for *D. batis* (*COIhap7*).

About 640 bp of nucleotide sequence for the CR
fragment from *D. oxyrinchus*, *Dipturus* *sp.*, *D. nidar-
osiensis* and *D. batis* individuals, both of Mediterra-
near and Atlantic origin, were determined.

Two different CR haplotypes were found for *D. nidarosiensis* (*CRhap1–2*), four CR haplotypes
were found for *Dipturus* *sp.* (*CRhap1–4*), two CR haplo-
types for *D. batis* (*CRhap 11–12*) and five CR
haplotypes for *D. oxyrinchus* specimens (*CRhap5,
7–10*). In particular, *CRhap8* and *CRhap7/9*
were identical to the homologous portion of the *D. oxyrinchus*
haplotypes by Valsecchi et al. (2005).

The sequencing of three mitochondrial fragments
(16SrDNA, CR and COI) showed that the *Dipturus*
specimens from the NE Atlantic and therefore they were classified as this species.

The three species of *Dipturus* (the common skate,
the longnose skate and the Norwegian skate) can be
easily discriminated by molecular means, since
16SrDNA, CR and COI species-specific haplotypes
were found for all of them. Nevertheless, the analysis
of sequences indicated that the three markers were
not informative in the same way. Both the CR and
COI fragments were considerably more variable than
the 16SrDNA. Numbers of differences and Kimura
(1980) genetic distances within and between species
are reported in Table III.

Overall, the Kimura mean genetic distances
among the three species of *Dipturus* were very low
for the 16SrDNA (DK2P% = 0.98%), low for the
COI sequences (DK2P% = 3.45%) and CR (DK2P% = 4.57%). In all cases the mean intra-
species divergence resulted in lower than mean
inter-species divergence. In particular, for the COI
sequences the mean interspecies divergence was
largely higher than 10 times the mean intra-specific
variation (DK2P% = 0.16%); the standard diver-
gence threshold to delimit species proposed by
Hebert et al. (2004), known as the ‘10-fold rule’,
was largely overcome.

Besides the distance between sequences, phyloge-
netic trees were also reconstructed to assess the
relationship between the *Dipturus* *sp*. sequences and
their neighboring sequences. All the phylogenetic
analyses realized for the three markers separately

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**Figure 2.** Comparison between *Dipturus nidarosiensis* (a) and *D. oxyrinchus* (b) diagnostic morphological features. In figures a1 and b1 the snouts of the two species are shown; this is clearly longer and more pointed in *D. oxyrinchus*. In figures a2 and b2 ventral sides are shown; the underside is uniformly dark in *D. nidarosiensis* while pale grey-whitish with black dots in *D. oxyrinchus*. Finally, figures a3 and b3 show the tail and the associated thorns; the number of medio-dorsal thorns is higher in *D. nidarosiensis*, in this species they are pointed and rearward oriented.
Table III. Genetic distances within (diagonal elements) and between species (below the diagonal). The distance values were computed with the Kimura (1980) model. Above the diagonal the number of differences and fixed differences (between parentheses) between species.

|          | D. nidarosiensis | D. oxyrinchus | D. batis |
|----------|------------------|---------------|---------|
| 16SrDNA  |                  |               |         |
| D. nidarosiensis | 0.00 | 5–7 (5) | 9–10 (9) |
| D. oxyrinchus    | 0.84 | 0.34 | 5–7 (4) |
| D. batis         | 1.52 | 0.67 | 0.17    |
| COI         |                  |               |         |
| D. nidarosiensis | 0.24 | 24–27 (24) | 32–33 (32) |
| D. oxyrinchus    | 4.57 | 0.24 | 19–23 (21) |
| D. batis         | 6.07 | 3.97 | 0.00    |
| CR          |                  |               |         |
| D. nidarosiensis | 0.23 | 46–50 (45) | 47–48 (45) |
| D. oxyrinchus    | 7.74 | 0.28 | 22–25 (22) |
| D. batis         | 7.77 | 3.57 | 0.16    |

(data not shown) produced trees with similar topologies where all specimens were assigned to the same clades. Dipturus sp. individuals clustered together with D. nidarosiensis specimens. Dipturus nidarosiensis, D. oxyrinchus and the Atlantic D. batis appeared as three well-separate clades, supported by high bootstrap values (Figure 3).

**PCR-RFPL**

The sequential digestion of CR PCR-products with the two endonucleases DdeI and TaqI resulted in species-specific pattern of fragments that can be used to distinguish the three species of *Dipturus*.

After digestion of the CR PCR-amplified fragments, *D. nidarosiensis*, *D. batis* and *D. oxyrinchus* DNAs could be readily distinguished through agarose gel electrophoresis (Figure 4).

**Discussion**

Morphologically, the Sardinian specimens appeared similar or identical to *D. nidarosiensis* and distinct from the other *Dipturus* species from the Mediterranean and eastern Atlantic.

In the past, the number of *Dipturus* species in the Mediterranean Sea was questioned and morphological discrimination between them has been regarded as a very difficult task for non-specialists because of the lack of readily scorable diagnostic characteristics. Doubts about the validity of historical identifications of *D. batis* in the Mediterranean region are reported (Dulvy et al. 2006), because this species could potentially be confused with *D. oxyrinchus*, despite morphological and colour differences (Tortonese 1956; Dulvy et al. 2006). Unfortunately, to the best knowledge of the authors, pictures are not available in referenced publications for any *D. batis* caught in the Mediterranean Sea. The only visual representation of Mediterranean *D. batis* specimens is the drawing by Tortonese (1956) (Figure 5a). Furthermore, from a preliminary survey in the European Museum and Ichthyologic Collections the voucher specimens collected in the Mediterranean and classified as *D. batis*...
were either *D. oxyrinchus* or *R. alba* (Cannas & Iglesias, pers. comm.). The occurrence of *D. batis* in the Mediterranean region is based only on catch data of trawl surveys (e.g. Medits and Grund surveys), whose accuracy in species-specific identification is somehow questionable.

Sequence divergence of the three regions of mtDNA sequenced (16SrDNA, control region and cytochrome c oxidase subunit I) indicated that Sardinian specimens were different from both the common skate, *D. batis* and the longnose skate, *D. oxyrinchus* but identical to *D. nidarosiensis* specimens.

Among the three mitochondrial markers, the 600 base pair 16SrDNA sequences of *D. nidarosiensis* exhibit very low sequence divergence from all other *Dipturus* species (0.84% from *D. oxyrinchus* and 1.52% from *D. batis*). The 560 base pair COI DNA ‘barcodes’ derived from specimens of *D. nidarosiensis* exhibit greater sequence divergence; the level of pairwise sequence divergence for *D. nidarosiensis/D. oxyrinchus* (4.57%) and *D. nidarosiensis/D. batis* (6.07%) was well above the threshold (10 × inter-/intra-specific divergence) proposed by Hebert et al. (2004) and Lefébure et al. (2006) for true species. The average COI sequence divergence between *Dipturus* spp. (DK2P = 3.45%) was about 20 times that within species; it was low relative to values reported among other chordates (Hebert et al. 2003) and fishes (Ward et al. 2009), but appears typical of other Chondrichthyes. For instance, the average COI sequence divergence between species was 5.5% and within species was 0.2% for the *Dipturus/Zearaja* complex (Ward et al. 2008).

Among species of *Bathyraja* average interspecies distance was even lower, 3.6% (Spies et al. 2006) and 2.9% for *Leucoraja erinacea* and *L. ocellata* (Alvarado Bremer et al. 2005). This very limited degree of separation could be explained by the well known slow nucleotide substitution rates in the COI gene in sharks, seven- to eightfold lower than in primates or ungulates (Martin et al. 1992; Martin & Palumbi 1993). Finally, the 640 base pair CR DNA ‘barcodes’ derived from specimens of *D. nidarosiensis* exhibit the greatest sequence divergence from all other *Dipturus* species (DK2P = 7.74% from *D. oxyrinchus* and DK2P = 7.77% from *D. batis*). Because of its high nucleotide polymorphism, the CR could represent a solid marker for species identification. Furthermore, based on the CR sequence variation among the three species of *Dipturus*, a very simple PCR-RFLP assay has been developed to routinely identify in the laboratory samples of adults, juveniles or eggs.

Taken together, these independent morphological and molecular observations serve to corroborate one another and thus provide strong evidence for the recognition of *D. nidarosiensis* as a new species in the Chondrichthyan fauna list for the Mediterranean area.

In the present study, the first record of the Norwegian skate in the Mediterranean Sea is reported. In view of the small sampling effort invested so far in deep areas of the Mediterranean Sea and the relatively few data known for the deep-sea biodiversity, the record of *D. nidarosiensis* in the
Sardinian waters represents an important contribution and allows for an update of the Mediterranean deep-sea elasmobranch biodiversity.

In reality, the presence of skates resembling to the Norwegian skate can be found in old books describing the Italian marine fauna. Doderlein (1885) listed among the species of Rajidae a third species other than the common skate ‘batis’ and the longnose skate ‘oxyrinchus’. This is described as a dark skate, brown both dorsally and ventrally, named *Raja macro-rhynchus* Rafinesque 1810, the same species is also included in Bonaparte (1832) as *Laeviraja macro-rhynchus* (Figure 5b).

Therefore, it is possible that representatives of the Norwegian skate could have been caught in the past but they were unnoticed.

The alternative hypothesis is that *D. nidarosiensis* could represent a new species, entered from the Atlantic in recent years. In fact, over 500 alien species (macrophyses, invertebrates and fishes) have been recorded thus far in the Mediterranean Sea (Galil 2007); however, the great majority of these records refer to coastal benthic species (Bianchi 2007). In effect, during interglacial periods, characterized by a temperate to warm climate as today, the superficial current entering the Mediterranean allows the colonisation of subtropical species from the Senegalese province while the outgoing bottom-current made the arrival of deep-sea denizens difficult (Emig & Geistdoerfer 2004). Therefore, the most likely hypothesis is that bathyhal *D. nidarosiensis* has inhabited the Mediterranean Sea at least since the last glacial period, when many benthal species from the Atlantic (of temperate/boreal origin) entered the basin, favoured by the inverted direction of the bottom-currents through the strait of Gibraltar (Emig & Geistdoerfer 2004).

Apart from speculations on the origin of the Norwegian skates in the Mediterranean, this paper confirms the effectiveness of molecular techniques for identification of species in a group of fish where morphological characters alone makes species identification difficult. Despite the known limited sequence divergence among species that in the past did not always allow for the discrimination among closely related skates (Valsecchi et al. 2005; Spies et al. 2006), mitochondrial sequences obtained here resulted in valid DNA markers for a clear separation of the species studied. The availability of DNA sequences and of an easy PCR-RFLP assay for the three species of *Dipturus* will be of great help in the future, assisting both in the accurate identification of adults of the genus and in the identification of eggs and young skates, whose morphological characters may not be fully reliable (Daan 2001).

### References

Alvarado Bremer JR, Frisk MG, Miller TJ, Turner J, Vinas J, Kwil K. 2005. Genetic identification of cryptic juveniles of little skate and winter skate. Journal of Fish Biology 66:1177–82.

Bertrand J, Gil De Sola L, Papacostantinou C, Relini G, Souplet A. 2002. The general specifications of the Medits surveys. Scientia Marina 66(2):9–17.

Bianchi CN. 2007. Biodiversity issues for the forthcoming tropical Mediterranean Sea. Hydrobiologia 580:7–21.

Bonaparte CL. 1832. Iconografia della Fauna Italica per le quattro classi degli animali vertebrati. Tomo III. Pesci. Roma: Salvucci.

Compagno LJV. 1999. Checklist of living elasmobranchs. In: Hamlett WC, editor. Sharks, Skates, and Rays: The Biology of Elasmobranch Fishes. Baltimore, MD: The Johns Hopkins University Press. 515 pp.

Daan N. 2001. The IBTS database: A plea for quality control. ICES CM:T-03.

Doderlein P. 1885. Manuale itiologico del Mediterraneo. Fascicolo III. Elasomobrachi, Batoidei. Palermo: Tipografia del giornale di Sicilia, Palermo. p 150–68.

Dulvy NK, Notobartolo di Sciara G, Serena F, Tinti F, Ungaro N, Mancusi C, et al. 2006. *Dipturus batis*. In: IUCN 2008. 2008 IUCN Red List of Threatened Species. Available from: www.iucnredlist.org. Accessed 15 May 2009.

Ebert DA, Compagno LJV. 2007. Biodiversity and systematics of skates (Chondrichthyes: Rajiformes: Rajoidoei). Environmental Biology of Fishes 80:111–24.

Emig CC, Geistdoerfer P. 2004. The Mediterranean deep-sea fauna: Historical evolution, bathymetric variations and geographical changes. Carnets de Geologie (1):1–10.

Galil BS. 2007. Loss or gain? Invasive aliens and biodiversity in the Mediterranean Sea. Marine Pollution Bulletin 55:314–22.

Gibson C, Valenti SV, Fordham SV, Fowler SL. 2008. The Conservation of Northeast Atlantic Chondrichthynans: Report of the IUCN Shark Specialist Group Northeast Atlantic Red List Workshop. viii +76pp.

Hebert PDN, Cywinska A, Ball SL, de Waard JR. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society B 270:313–22.

Hebert PDN, Stoeckle MY, Zemla TS, Francis CM. 2004. Identification of birds through DNA barcodes. PLoS Biology 2:1657–63.

Hubbs CL, Ishiyama R. 1968. Methods for the taxonomic study and description of skates (Rajidae). Copedia 1968:483–91.

Ishihara H. 1987. Revision of the western North Pacific species of the genus *Raja*. Japan Journal of Ichthyology 34:241–85.

Johnston G, Figueuredo I, Machado P, Clarke M, Bladssdale T, Ellis J, et al. 2005. Separation of species data from national landing figures. ICES Document CM 2005/N: 22. 16 pp.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111–20.

Lefebure T, Douad CJ, Gouy M, Gibiert J. 2006. Relationship between morphological taxonomy and molecular divergence within Crustacea: Proposal of a molecular threshold to help species delimitation. Molecular Phylogenetics and Evolution 40:435–47.

Martin AP, Naylor GJP, Palumbi SR. 1992. Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. Nature 357:153–55.

Martin AP, Palumbi SR. 1993. Protein evolution in different cellular environments: Cytochrome b in sharks and mammals. Molecular Biology and Evolution 10:873–91.
