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Genetic identification of cryptic juveniles of little skate and winter skate

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As the morphological discrimination of winter skate *Leucoraja ocellata* and little skate *Leucoraja erinacea* juveniles is unreliable, a genetic assay, based on the restriction digest with *Sty* I of a segment of mitochondrial DNA cytochrome oxidase I gene, capable of discriminating the species was developed. The reliable identification of species can be used to improve the accuracy of population assessment models.

Key words: *Amblyraja radiata*; forensic identification; genetic differentiation; *Leucoraja erinacea*; *Leucoraja ocellata*; skates.

There is an urgent need for development and implementation of management polices for western Atlantic skates (Rajidae) because the winter skate *Leucoraja ocellata* (Mitchell), thorny skate *Amblyraja radiata* (Donovan) and barndoor skate *Dipturus laevis* (Mitchell), are over-fished (NEFSC, 2000). Winter skate and little skate *Leucoraja erinacea* (Mitchell), are abundant skate species in the western Atlantic and are taken as commercial, recreational and by-catch harvests (McEachran, 2002). Efforts to develop management strategies for winter and little skate have been hampered because of a paucity of information on vital rates, which are not well known because it is difficult to differentiate juveniles (<30 cm) of the two species (McEachran & Musick, 1973). Accordingly, the National Marine Fisheries Service's (NMFS) fishery-independent surveys do not estimate abundances for small length classes for winter and little skate separately. Previous studies have relied on morphological identification or excluded young age groups (Waring, 1984). Molecular techniques such as the polymerase chain reaction-restriction fragment length polymorphism

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(PCR-RFLP) have proved useful to discriminate morphologically similar fishes (Chow & Inoue, 1993; Alvarado Bremer *et al.*, 1998). Accordingly, to facilitate the identification of juveniles of winter skate and little skate, a rapid PCR-RFLP assay was developed based on the restriction activity of the endonuclease *Sty* I on an amplified segment of the mitochondrial DNA (mtDNA) cytochrome oxidase I (CO I) gene. This assay was developed as follows: sequences from adult individuals of both species were characterized and the fixed nucleotide differences were used to identify potential diagnostic restriction assays. After preliminary screening, the assay that produced the most distinct restriction pattern was selected. This assay was then tested on a sample of known adult specimens, followed by screening of a sample of unknown juveniles. The species identification obtained with the PCR-RFLP assay was verified by direct sequencing. The development of a reliable method to differentiate between juveniles of these two species will lead to the collection of data that can aid in future stock assessments of little and winter skate in the western Atlantic.

Winter skate and little skate were obtained along the U.S. east coast during the NMFS's annual fishery-independent trawl surveys (autumn 1999, 2001; winter 2002; spring, 1999, 2002) and sea scallop *Plactopecten magellanicus* (summer, 2001) surveys aboard the RV Albatross IV and from commercial fishermen off Ocean City, MD, U.S.A. Little and winter skate sizes in the collections ranged from 9–57 and 16·5–107 cm total length (L_T), respectively. Skates >30 cm L_T can be reliably differentiated based on morphological characteristics (McEachran, 2002). Tissue samples (heart or axial muscle) were removed from specimens and frozen at −76° C until assayed. Total DNA was isolated as described in Greig (2000). By comparing the mtDNA sequence of thorny skate (Gene Bank Accession number NC_000893; Rasmussen & Arnason, 1999) against the universal primers CO9 (6607) and CO10 (7214) (Baldwin *et al.*, 1998), two new skate-specific primers were designed: the forward primer RajaCOIf (5′-CTTTGGTCACCTGAAGTATAT-3′) and reverse primer RajaCOIr (5′-TAAGCATCTGGGTAGTCTGAATAGCG-3′). Thermocycling conditions were as follows: an initial denaturing step at 94° C for 3 min, followed by 35 cycles of denaturing at 94° C for 45 s, annealing at 55° C for 45 s, and extension at 72° C for 1 min, followed by a single extension step at 72° C for 5 min.

Prior to sequencing, primers and unincorporated nucleotides were removed from PCR products using the ExoSapIt kit (USB, Cleveland OH, U.S.A.) following the manufacturer's recommendations. Cycle sequencing reactions were performed in 10 µl volumes consisting of 4 µl of purified PCR template and 6 µl of BigDye terminators (ver. 2.0; Applied Biosystems Foster City, CA, U.S.A.) as recommended by the manufacturer. Unincorporated fluorescent-nucleotides were removed from sequencing reactions using RapidXtract Dye Terminator Removal Kit (Prolinx, Bothell, WA, U.S.A.). Exactly 20 µl dionized water were added to each reaction prior to capillary electrophoresis. Sequencing reactions were analysed on an ABI 310 Genetic Analyzer (Applied Biosystems) in both directions using the forward and reverse primers. Sequences were aligned in BioEdit, version 5.0.9 (Hall, 1999) against the published sequence of thorny skate. Diversity indices including haplotypic diversity (h) (Nei & Tajima, 1981; Nei, 1987), the average number of pairwise nucleotide substitu-

tions ($k \pm$ S.D.) (Tajima, 1983), and the number of segregating sites (S) were estimated as implemented in ARLEQUIN (Schneider *et al.*, 2000). The net number of nucleotide substitutions per site ($D_A \pm$ S.E.) (Nei, 1987) between species was estimated with the computer package DNA Sequence Polymorphism (DnaSP; Rozas & Rozas, 1997) using Jukes & Cantor (1969) distances. Restriction sites were identified from aligned sequences with the programme Web Cutter invoked in BioEdit. Approximately 5 µl of PCR product were digested with *Sty* I following the manufacturer's recommendations (New England Biolabs, Beverly, MA, U.S.A.) in 10 µl volumes. Electrophoresis of entire digestion reactions was carried out in 1·5% agarose Tris-acetate (TA) gels to characterize size polymorphisms.

Amplification of skate CO I mitochondrial gene yielded a single PCR fragment *c.* 629 bp long. A total of 498 bp of nucleotide sequence of this fragment was determined from 10 little skates and 11 winter skates. There were no insertions or deletions among the sequences. A comparison of the 21 little and winter skate sequences showed that 29 (5·8%) of the sites were segregating (polymorphic) (Fig. 1). These polymorphisms included 12 fixed differences separating winter from little skate, and included 10 transitions and two transversions. Within species, little skate sequences contained 13 segregating sites, and winter skate sequences seven. Accordingly, the value of k was almost twice as large for little skate ($3{\cdot}45 \pm 1{\cdot}92$) than for winter skate ($1{\cdot}82 \pm 1{\cdot}31$). Haplotypic diversity in little skate equalled unity, since all individuals had unique

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                              *
                     111 1122222233 3333334444 444
                  123678012 6601245712 3377790022 478
                 5792873138 1903787519 7923973414 325
Reri009          CAACACAGTC ATCAACTGCC GCTATCACTT GCA
Reri010          T.G....... .........T T....T.T.. ...
Reri011          T..T...... .......... T......T.. ...
Reri012          T..?...... .C........ T......T.. ...
Reri016          T......... .......... T.?....T.. ...
Reri-017-Sum01   T......... .......... TG..C..T.. ...
Reri-009-Sum01   T.....G... .......... TG.....T.. ...
Reri-U14-12-00   T..T...T.. .......... TG.G...T.. ...
Reri-U16-12-00   T..T...... .......... TG.....T.. ...
Reri-U17-12-00   T?G....... .........T TG...T.T.C ...
Roce005          T.GT.T..AT G.T..TAAT. T.G.C.GTC. AT.
Roce008          T.GT.T..AT G.T.TTGAT. ....C..TC. AT?
Roce009          TTGT.T..AT G.T..TGAT. T...C..TC. AT.
Roce010          T.GT.T..AT G.T..TAAT. T...C..TC. AT.
Roce011          T.GT.T..AT G.T..TAAT. T...C..TC. AT.
Roce012          T.GT.T..AT G.TT.TGAT. T...C..TC. ATG
Roce015          T.GT.T..AT G.T..TAAT. T...C..TC. AT.
Roce017          T.GTGT..AT G.T..TGAT. T...C..TC. AT.
Roce018          T.GT.T..AT G.T..TGAT. T.?.C..TC. AT.
Roce019          T.GT.T..AT G.T..TAAT. T...C..TC. AT.
Roce022          T.GT.T..AT G.T..TGAT. T...C..TC. AT.
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FIG. 1. Polymorphic sites contained within the 498 bp segment of the mitochondrial CO I gene from representative little skate and winter skate specimens. Identical bases (.) and ambiguous positions (?) are shown. The first nucleotide in this study corresponds to nucleotide position 6308 of the complete mtDNA genome of thorny skate. *, the location of the polymorphism responsible for the presence or absence of the *Sty* I restriction site. DNA sequences have been submitted to GenBank (Accession numbers AY573823–AY573843).

haplotypes; in winter skate, two haplotypes were repeated, one four times (Roce010, Roce011, Roce015 and Roce019) and the second two times (Roce018 and Roce022), to give a value of haplotypic diversity of 0·87. The marked differences in the amounts of genetic variation contained in the samples of these two species are evident in the neighbour joining (NJ) gene-tree (Fig. 2). Sampling bias appears not to account for such disparity, particularly since the less variable winter skate sample came from a wider geographical area than the little skate sample that was collected primarily off the coast of Maryland. The NJ-tree also reveals the close phenetic relationship of winter and little skates. The corrected mean difference (D_A) between winter skate and the little skate samples was 2·9%. By contrast, the value of D_A values separating little skate

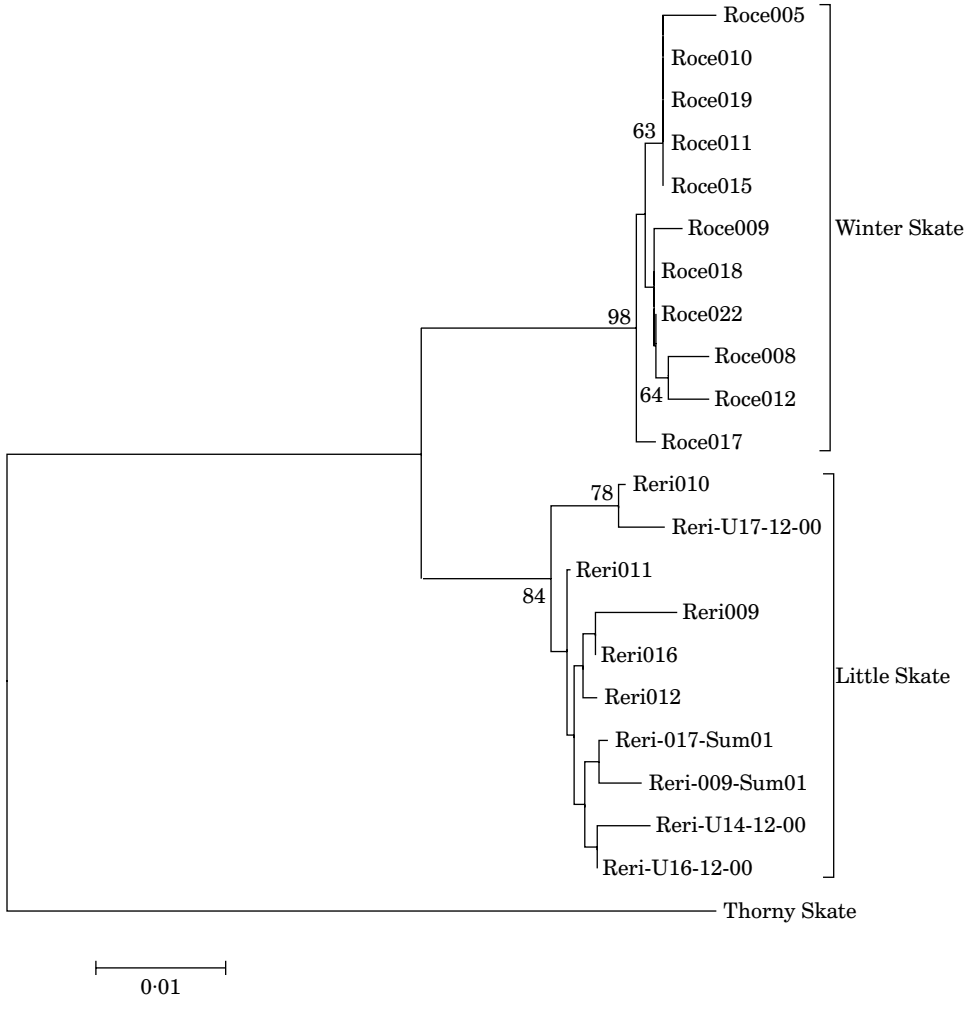

FIG. 2. Neighbour joining (Saitou & Nei, 1987) gene-tree based on Tamura & Nei (1993) distances showing the relationship among little skate and winter skate using thorny skate (GenBank Accession NC_000893) as the outgroup. Values at nodes represent proportion of bootstrap support ≥60%.

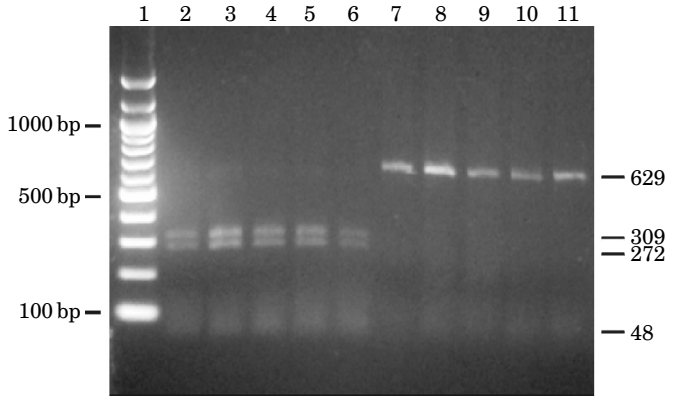

FIG. 3. Agarose gel (1·5%) of winter skate and little skate mtDNA CO I digested with restriction endonuclease *Sty* I. Lane 1, 100 bp ladder (New England Biolabs); lanes 2–6, winter skate DNA; lanes 7–10 little skate DNA; lane 11 uncut winter skate DNA. The values to the right of the gel correspond to the fragment sizes (bp) generated by the restriction digest.

and winter skate from thorny skate were three times larger at 8·9 and 9·2%, respectively.

One of the diagnostic differences distinguishing little skate from winter skate corresponded to a C-T substitution at nucleotide position 200 (Fig. 1), which was part of the recognition site for *Sty* I in winter skate (CCATGG) that was absent in both little skate and thorny skate (CCACGG). In addition, there was a second C-T substitution (unpubl. data), located eight nucleotides downstream from the 3′ end of the COI segment characterized in this study, which generated a *Sty* I site in winter skate and in the reference sequence of thorny skate, but not in little skate.

To determine if the *Sty* I site could be used to distinguish the mtDNA of winter and little skate, additional adults were screened for the presence of the *Sty* I site. The DNA of all winter skate ($n = 29$) specimens were cut but none of the little skate CO I fragments ($n = 26$) were cut. The two haplotypes could be readily distinguished through agarose gel electrophoresis (Fig. 3). Using this approach, samples of unknown juveniles can be routinely identified in the laboratory. Thus, a very simple assay has been developed to discriminate between mtDNAs from little and winter skate.

The capability of discriminating between DNA samples of winter skate and little skate using a simple PCR-RFLP assay offers the potential of a reliable and robust identification of the cryptic juveniles of these skate species. This technology could be used during the NOAA Fisheries surveys to collect information for estimating juvenile growth, abundance and recruitment for winter and little skate and ultimately improve management polices.

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