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Characterization of a New Molecular Marker for Investigating Skate Population Genetics: Analysis of Three Mediterranean Skate Species (genus *Raja*) of Commercial Interest as a Test Case

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Abstract

In this study we describe a new set of primers specifically designed for the amplification of the mitochondrial control region in skates of the Rajinae subfamily. The suitability of the amplified region as molecular marker was tested on three Mediterranean skates species of commercial interest (*Raja clavata*, *Raja asterias* and *Raja miraletus*). Fifty-six skate samples collected from 6 locations around the Italian peninsula were molecularly typed for 324–332bp of the mitochondrial DNA control region. The target sequence was polymorphic and, therefore, informative in all three species. Although the number of individuals screened for each species/location was small, our data suggest that populations of *R. clavata* and *R. miraletus* in central Mediterranean are single stocks. Conversely, there may be two genetically differentiated forms of *R. asterias*: one in the Adriatic Sea and the other in the Ligurian/Tyrrehenian Seas which represent separate geographic units or stocks. The highest level of genetic variability was recorded for *R. clavata* ($H = 0.61$) and the lowest for *R. miraletus* ($H = 0.17$), with *R. asterias* showing an intermediate level ($H = 0.29$), though no variation was found within the two (Adriatic and Ligurian/Tyrrehenian) sub-populations of the latter. These preliminary data show the potential of the described primers set to be employed in skate population genetics and phylogeny.

Key words: D-loop, elasmobranch, genetics, mtDNA, rajinae, skate

Introduction

The skate and ray (Rajidae) family has high species diversity (>250 species) and is found worldwide in marine waters, from shallow benthic habitats to abyssal regions (e.g. McEachran and Miyake 1990). Despite showing some interesting biological features, such as the extremely high morphological conservatism across present and fossil species (Capetta, 1987), and despite having proved to be highly susceptible to fishery pressure in many oceanic districts (e.g. Brander 1981; Casey and Myers, 1998; Dulvy *et al.* 2000; Walker and Hislop 1998; Aldebert 1997), skates still remain poorly studied. Most studies on elasmobranch population structure have relied on fishery catch data (e.g. Fitzmaurice 1974, Aldebert 1997) or on tagging programs (e.g. Templeman 1984, Little 1995, Duarte *et al.* 2002) to determine species distribution, abundance or dispersal potential and to detect demographic fluctuations. However, these approaches lack the resolution to assess population sub-structuring (e.g. definition of stocks) or to clarify the phylogenetic relationship among species.

In the last two decades, molecular techniques have been used to address these aspects in a wide range of animal populations, including many fish species (e.g. Rocha-Olivares *et al.* 2000; Takeyama *et al.* 2001), but this approach has not yet been employed in skates. One limitation could have been the lack of suitable markers specifically designed for this group of animals. Moreover, to be informative for population genetics purposes, molecular markers should be variable (i.e. polymorphic) at the species level, a characteristic which might be expected to be rare in animals with such a low morphological diversity.

In this study we describe a new set of PCR (polymerase chain reaction) primers specifically designed for the amplification of the hyper variable region 1 (HVR1) of the mitochondrial control region (the most variable portion of the mitochondrial genome) in skates of the Rajnae subfamily. We tested the efficiency (level of polymorphism) of the isolated sequence as a molecular marker in three skate species (*R. clavata*, *R. asterias* and *R. miraletus*).

Materials and Methods

Design of D-loop primers

A new set of three primers was designed to obtain PCR amplifications of the D-loop region (the most variable part of the mtDNA control region) from members of

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the subfamily Rajinae. The light-strand primer, ElDloopF (5'-TCCCAAAGCCAAGATTCTGC-3'), was designed from a region of the $tRNA^{Pro}$ gene which was found to be highly conserved across most Elasmobranch species for which mitochondrial DNA sequences are available from GenBank (*Amblyraja radiata*, GenBank a.n.: AF106038; *Mustelus manazo*, GenBank a.n.: AB015962; *Squalus acanthias*, GenBank a.n.: Y18134; *Heterodontus francisci*, GenBank a.n.: AJ310141; *Scyliorhinus canicola* GenBank a.n.: Y16067). No equally conserved region was found on the heavy strand. We therefore designed a reverse primer, RajinaeP7r (5'-AAACTGGGAGGGCTGGAAATCTTGA-3'), on the *A. radiata* sequence in the homologous region of Primer-7 described by Kitamura *et al.* (1996). Primers ElDloopF and RajinaeP7r produced a PCR product of approximately 588bp in the three tested species and in *Dipturus oxyrinchus*. These fragments were cloned and sequenced in the four species. From the largest conserved region identified in the four cloned sequenced we designed a third (heavy strand) internal primer (DLRajaR, 5'-CCATACACACATGTGTAATGAAATAGTTG-3') which, paired with ElDloopF, allows the amplification of a shorter (approximately 390bp) and therefore more convenient fragment to be analysed and sequenced.

Samples and DNA preparation

The newly designed primers were tested on a panel of 26 *R. clavata*, 12 *R. miraletus* and 18 *R. asterias* specimens collected in 2000 and 2001 from six sampling locations around the Italian coast (Fig. 1). *R. clavata* samples were collected from all six locations, while *R. miraletus* and *R. asterias* were only found at 3 and 4 sites respectively. For each species the two sexes were equally represented. We also had access to a sample of *R. clavata* from the Atlantic Ocean (Celtic Sea). In the Mediterranean, *R. clavata* exhibits morphological variation with a still unknown phylogenetic relationship between different morphotypes, and we therefore used only *R. clavata* samples which could be unambiguously assigned to this species.

DNA was extracted from muscle samples excised from the ventral side of the pectoral fins of fresh or frozen skates. The tissue samples were preserved in a solution of 20% DMSO (dimethylsulphoxide) saturated with NaCl (Seutin *et al.*, 1991) until extraction. This solution allows tissues to be preserved at room temperature for long periods of time, though we stored the samples at 4°C in the laboratory. DNA was extracted using standard procedures (Sambrook *et al.*, 1989).

DNA amplification and sequence of the D-loop region

For all PCR reactions we used the following reaction mix: 10ng of genomic DNA, 2.5µl 10x Taq Buffer (Promega), 1.5µl $MgCl_2$ (25mM), 1µl of each primer (10µM), 0.3µl dNTPs (10µM), 0.2µl Taq DNA Polymerase (Promega). Each PCR sample was brought up to 25µl with sdH_2O. Where it was necessary to optimise PCR specificity (see below), 1–2% DMSO was included in the mix. This compound increases band clarity and decreases the number of spurious bands.

The reactions were covered with 15µl of mineral oil to avoid evaporation during thermal cycling. We used slightly different PCR profiles for the two primer combinations. For the amplification with primers ElDloopF-RajinaeP7r, the PCR profile consisted of the following steps: a 3 min denaturation (93°C) phase followed by 35 cycles each consisting of denaturation (92°C, 35 sec), annealing (43°C, 55 sec) and extension (72°C, 75 sec) phases; the programme terminated with a final extension step of 7 min at 72°C. For the amplification with primers ElDloopF-DLRajaR the 35 cycles consisted of denaturation (92°C, 10 sec), annealing (55°C, 10 sec) and extension (72°C, 30 sec) steps. This second combination of primers required more stringent conditions as primer DLRajaR has high homology (75.9%) for a DNA region included between the two annealing sites of primers ElDloopF and DLRajaR. When combined, these two primers may amplify a secondary band (of about 195bp), which may be avoided by keeping high annealing temperature or by adding DMSO (1–2%) to the PCR mix.

Amplification products were sequenced using BigDye™ (Perkin-Elmer Corporation) sequencing mix, on a ABI-377 automated sequencer. The sequencing reaction was performed using the following conditions: a 3min denaturation at 98°C followed by 25 cycles each consisting of denaturation (96°C, 30 sec), annealing (50°C, 10 sec) and extension (60°C, 2 min, 30 sec.). After cycle sequencing the reactions were purified using the ExoSAP-IT™ (USB Corporation) protocol followed by precipitation of the sequencing product. This was then resuspended in 16µl of sdH_2O and loaded on the automatic sequencer.

Analysis of molecular data

For each species, geographical differentiation among the various regional populations was quantified from the distribution of haplotypes using both analysis of molecular variance (AMOVA, Excoffier *et al.* 1992), which takes into account information on nucleotide differences (Φ_{ST}), as well as the traditional ANOVA based on the correlation of a random pair of haplotypes within a population relative to the total population (conventional F_{ST} statistic, Wright, 1951). While F_{ST} considers unique differences in haplotype frequencies, the Φ_{ST} statistic also incorporates a measure of the molecular distances (in our case pairwise number of substitutions) between haplotypes. Statistical

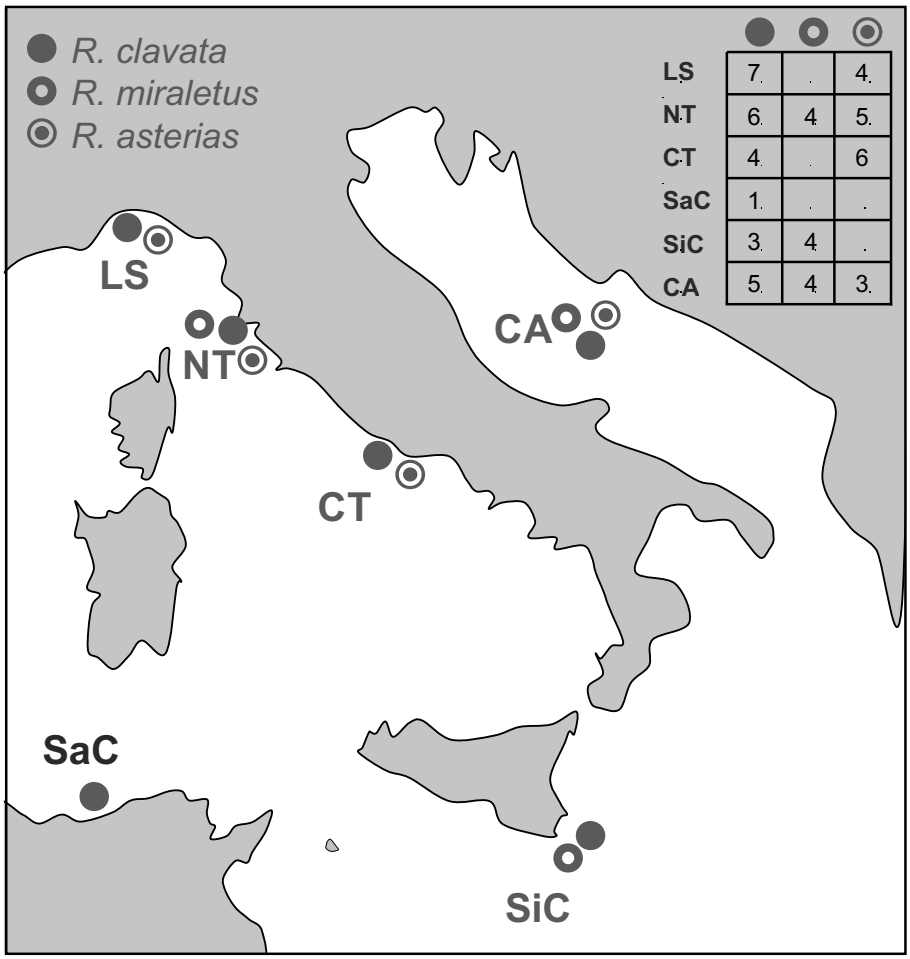

Fig. 1. Sampling sites: Ligurian Sea (LS); Northern Tyrrhenian Sea (NT); Central Tyrrhenian Sea (CT); Sardinian Channel (SaC); Sicilain Channel (SiC); Central Adriatic Sea (CA). Sample sizes are shown at top right-hand corner.

significance was tested using a permutation procedure and 10 000 replications. We also performed an exact test for population differentiation, which tests the hypothesis of random distribution of the individuals between pairs of populations (Raymond and Rousset, 1995). Exact tests are advisable when the actual allele frequencies in the populations are unknown and/or sample sizes are particularly small, as in our case. Significance was estimated using the Markov Chain algorithm with 10 000 steps (Raymond and Rousset, 1995). Both calculations on population differentiation were performed using the computer program Arlequin v2.000 (Schneider *et al.*, 2000). Single population samples were excluded from the analyses.

Results

The newly designed set of PCR primers successfully amplified the D-loop region in three skate species, and the amplified fragment was sequenced in 56 samples from 3 to 6 different central Mediterranean locations for each species. The consensus segment of the mtDNA control region was found to be polymorphic in all three surveyed species.

Raja clavata

The analysed DNA fragment (335 bp) included 6 variable sites defining six unique sequences ($n = 26$; Table 1). Nucleotide diversity (π), i.e. the percentage of the average number of nucleotide differences per site between two sequences (Nei, 1987) was $\pi = 0.72\%$, while the haplotype diversity (H) was 0.61. This measurement is analogous to the heterozygosity for diploid data.

Two haplotypes, H01Rcl and H02Rcl, were fairly common within the whole sample ($p = 0.58$, $p = 0.27$ respectively) and were encountered in most of the surveyed regions (see Table 1). The Atlantic specimen carried one of these haplotypes (H02Rcl). The remaining four

TABLE 1. MtDNA haplotype distribution in (**A**) *R. clavata*, (**B**) *R. asterias*, and (**C**) *R. miraletus* from different locations (see Figure 1) within central Mediterranean. The second column indicates the nucleotide positions (to be read vertically) of variable sites: dots (.) denote identity at the corresponding position in haplotype H01Rxx. The complete DNA sequences of haplotypes H01Rcl, H01Ras and H01Rmi are available from GenBank (a.n. AY167922- AY167924). Note that, in *R. clavata*, the DNA fragment included between positions 127 and 143 could not be unambiguously sequenced in most individuals and was therefore removed from the alignment and the analysis.

(**A**)

R. clavata													
	1	6	7	7	2	4							
Haplotypes	2	6	5	2	3	0	LS	NT	CT	SiC	SaC	CA	ATL	Total
H01Rc1	G	C	G	T	C	C	5	4	3			3		15
H02Rc1	A	.	.	.	.	.	2	1	1	1	1		1	7
H03Rcl	.	.	.	C	T	.						1		1
H04Rc1	A	.	.	.	.	T		1						1
H05Rc1	.	T	.	.	T	.						1		1
H06Rc1	.	.	C	.	.	.				1				1
							7	6	4	2	1	5	1	26

(**B**)

R. asterias	1	2	2				
	0	2	4				
Haplotypes	2	8	5	LS	NT	CT	CA	Total
H01Ras	C	C	C	4	5	6		15
H02Ras	T	T	T					3
				4	5	6	3	18

(**C**)

R.miraletus					
	5				
Haplotypes	1	NT	SiC	CA	Total
H01Rmi	A	4	3	4	11
H02Rmi	G		1		1
		4	4	4	12

haplotypes were found only in one specimen ($p = 0.04$). Two of these rare haplotypes, H03Rcl and H05Rcl, differed from the two commonest haplotypes for 2 variable sites (while the two commonest alleles differ from each other for only 1 variable site). Due to the small numbers in our test-samples, results on population differentiation are not robust, as apparent private alleles (i.e. alleles found exclusively in one location), which tend to inflate the estimation of genetic differentiation, may not actually be such when larger samples are considered. In order to decrease this effect, we performed the analysis for population differentiation both including and excluding rare alleles (Table 2 a,b). The exact test showed no geographic differentiation between the surveyed regions regardless of the inclusion/exclusion of rare haplotypes. However, the Φ_{ST} values indicated a significant differentiation between *R. clavata* samples of the Ligurian and the Adriatic seas, but only when rare alleles were included in the analysis. This might be due to two of the rare alleles being simultaneously present in the Adriatic Sea.

Raja miraletus

The analysed DNA fragment (330 bp) included 1 variable site defining two unique sequences ($n = 12$; Table 1). Nucleotide and haplotype diversities were $\pi = 0.31\%$ and $H = 0.17$ respectively. One of the two haplotypes,

TABLE 2. Results of population differentiation (see site locations in Fig. 1) calculated for: (**A**) *R. clavata* using all haplotypes; (**B**) *R. clavata* removing rare haplotypes; (**C**) *R. asterias* and (**D**) *R. miraletus*. Below the diagonals are Φ_{ST} values (underlined when significant, p <0.005); above the diagonal are the exact test P-values.

(**A**)

R. clavata	LS	NT	CT	CA
LS		1.000	1.000	0.368
NT	-0.12		1.000	1.000
CT	-0.24	-0.19		1.000
CA	0.17	0.14	0.07	

(**B**)

R. clavata	LS	NT	CT	CA
LS		1.000	1.000	1.000
NT	-0.18		1.000	1.000
CT	-0.24	-0.28		1.000
CA	-0.01	-0.13	-0.09	

(**C**)

R. asterias	LS	NT	CT	CA
LS		0.991	0.991	0.018
NT	0.00		0.991	0.018
CT	0.00	0.00		0.009
CA	1.00	1.00	1.00	

(**D**)

R. miraletus	NT	SiC	CA
NT		0.991	0.991
SiC	0.00		0.991
CA	0.00	0.00	

H01Rmi, was present in all but one individual (p = 0.92) and in all three sampling locations. The individual carrying haplotype H02Rmi was very small, but this species has unambiguous morphological features which make species misidentification unlikely even at young age. Besides, the low degree of sequence divergence between the two haplotypes (0.31%) is compatible with inter-specific variation. Our (limited) data showed no evidence for geographic differentiation among the surveyed regions with either of the statistical estimators used.

Raja asterias

The 329bp DNA-fragment sequenced in *R. asterias* individuals revealed 3 variable sites defining two unique sequences (n = 18; Table 1). Nucleotide and haplotype diversities were π = 0.92% and H = 0.29 respectively. One of the two haplotypes, H01Ras, was exclusively found in the individuals sampled from waters off the western coast of Italy (LS, NT and CT), while haplotype H02Ras was exclusively found in the individuals caught in the Adriatic sea (CA). Genetic differentiation between the two groups was highly significant.

Use of primers in related species

The homologous mtDNA control region sequence was also obtained for specimens of the genera *Leucoraja* and *Dipturus* using a combination of the three primers developed in this study. Specimens of the genus *Leucoraja* could not be amplified using the set of primers ElDloopF-DLRajaR. This was due to the annealing site of primer DLRajaR not being highly conserved within the Rajinae subfamily (e.g. only 27.6% homology in *Amblyraja radiata*). However, the genus *Leucoraja* could be amplified using the other primers' combination (ElDloopF- RajinaeP7r).

Discussion

Suitability of the D-loop region as molecular marker in stakes

The three primers developed in this study successfully amplified homologous sequences in a variety of Rajinae species, including 3 different genera. This region was found to be high variability across species (up to 44.4%), and may prove highly suitabley for taxonomic purposes and for clarifying taxonomic identity whenever species classification is uncertain based on morphological identification.

Population structure

Although our sample sizes were too small to perform a sensitive analysis of population differentiation (population screening was mainly performed to test level of inter-specific polymorphism in the isolated mtDNA sequence), we found little or no evidence of population structure for *R. clavata* and *R. miraletus* within the study area. The most likely explanation is extensive gene flow across the relatively low geographic distances between the surveyed areas. Interestingly, the *R. clavata* sample available from the Atlantic ocean carried one of the commonest haplotypes (H02Rcl) found in our Mediterranean samples, suggesting that this species might disperse far more than our geographic sample-range allowed us to test.

Our data revealed a different scenario for *R. asterias*, which seems to be present in two geographically and genetically distinct forms in central Mediterranean, with no evidence of gene flow between the two stocks. This species inhabits inshore waters (up to about 100 m deep; Stehmann and Bürkel, 1984), and it is possible that the discontinuity of the continental platform prevents *R. asterias* dispers-

ing over long distances, thus enhancing the formation of differentiated stocks. The relatively high sequence divergence ($\pi = 0.92\%$, higher than that found among the six *R. clavata* haplotypes) between the two haplotypes each characteristic of one of the two stocks, would suggest a long-term isolation between the two stocks.

Conservation and population status

Among the examined species, *R. clavata* revealed the highest genetic variability ($H = 0.61$), even when rare alleles ($p < 0.05$) were removed ($H = 0.45$). This is probably in agreement with the high morphological diversity that characterises this species (Stehmann and Bürkel, 1984). This phenotypic variability does not seem to reflect the species geographic distribution: within our study area different morphotypes live sympatrically.

Much lower levels of genetic polymorphism were recorded with *R. asterias* ($H = 0.29$) and *R. miraletus* ($H = 0.17$). Within each group of the Adriatic and Ligurian/Tyrrehenian *R. asterias*, there was no genetic variability. This might denote a recent bottleneck and suggests species/population vulnerability. If this is confirmed in future studies, both *R. miraletus* and *R. asterias* may require some management effort to ensure their preservation in central Mediterranean. Besides including larger sample sets, future works should also integrate data from nuclear (biparental) markers. This will allow to estimate the contribution of both sexes (e.g. sex-biased dispersal) in shaping the extant population structure.

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References

ALDEBERT, Y. 1997. Demersal resources of the Gulf of Lions (NW Mediterranean). Impact of exploitation of fish diversity. *Vie Milieu*, **47**: 275–284.

BRANDER, K. 1981. Disappearance of common skate *Raja batis* from the Irish Sea. *Nature*, **290**: 48-49.

CAPETTA, H. 1987. Chondrichthyes II. Mesazoic and Cenozoic Elasmobranchii. *In*: Handbook of paleoichthyology. H. P. Shultze (ed.). Gustav Fisher Verlag, New York, 193 p.

CASEY, L. M., and R. A. MYERS. 1998. Near extinction of a large, widely distributed fish. *Science*, **281**: 690–692.

DUARTE P. N., A. SILVA , and G. M. MENEZES. 2002. First results of a tagging program on tope shark, *Galeorhinus galeus*, and thornback ray, *Raja clavata*, in Azorean waters. 4th Meeting of the European Elasmobranch Association Proceedings. M. Vacchi, G. La-Mesa, F. Serena, and B. Seret (eds.) Paris France Societe francaise d'Ichtyologie, p. 197.

DULVY N. K., J. D. METCALFE, J. GLANVILLE, M. G. PAWSON, and J. D. REYNOLDS. 2000. Fishery stability, local extinctions, and shifts in community structure in skates. *Conserv. Biol.*, **14**: 283–293.

EXCOFFIER, L., P. E. SMOUSE, and J. M. QUATTRO. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**: 479–491.

FITZMAURICE, P. 1974. Size distribution and food of thornback rays (*Raja clavata* L.) caught on rod and line on the Mayo coast. Dublin-Ireland Stationery Office, *Ir. Fish. Investig. Ser. B*, No. 11.

KITAMURA, T., A. TAKEMURA, S. WABATE, T. TANIUCHI, and M. SHIMIZU. 1996. Mitochondrial DNA analysis for the cytochrome *b* gene and D-loop region from bull shark *Carcharhinus leucas*. *Fish. Sci.*, **62**: 21–27.

LITTLE W., 1995. Common skate and tope: First Results of Glasgow Museum's tagging study. *Glasgow Naturalist* **22**: 455–466.

McEACHRAN, J. D., and T. MIYAKE. 1990. Phylogenetic interrelationships of skates: a working hypothesis (Chondrichthyes: Rajoidei) *In*: Elasmobranchs as living resources: advances in the biology, ecology, systematics, and the status of the fisheries. H. L. Pratt, Jr., S. H. Gruber , and T. Taniuchi (eds.). *NOAA Technical Report*, **90**: 285–304.

NEI, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York, NY, USA, 512 p.

RAYMOND, M., and F. ROUSSET. 1995. An exact test for population differentiation. *Evolution*, **49**: 1280–1283.

ROCHA-OLIVARES, A., N. M. GARBER, and K. C. STUCK. 2000. High genetic diversity, large inter-oceanic divergence and historical demography of the striped mullet**.** *J. Fish Biol.,* **57**: 1134–1149.

SAMBROOK J., E. F. FRITSCH, and T. MANIATIS. 1989. Purification of Nucleic Acid in Molecular Cloning: a Laboratory Manual. 2nd editon. J. Sambrook, E. F. Fritsch, and T. Maniatis (eds.). Vol. 3, p. E3–E4, Cold Spring Harbour Laboratory Press, New York.

SCHNEIDER S., D. ROESSLI, and L. EXCOFFIER. 2000. Arlequin ver. 2.000: A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

SEUTIN, G., B. N. WHITE, and P. T. BOAG. 1991. Preservation of avian blood and tissue samples for DNA analysis. *Can. J. Zool.* **69**: 82–90.

STEHMANN, M., and D. L. BÜRKEL. 1984. Rajidae. *In*: Fishes of the north-eastern Atlantic and Mediterranean. P. J. P. Whitehead, M. L. Bauchot, J. C. Hureau, J. Nielsen, and E. Tortonese (eds.). UNESCO, Paris, vol.1, 163–196.

TAKEYAMA, H., S. CHOW, H. TSUZUKI, and T. MATSUNAGA 2001. Mitochondrial DNA sequence variation within and between tuna *Thunnus* species and its application to species identification. *J. Fish Biol.,* **58**: 1646–1657.

TEMPLEMAN, W. 1984. Migrations of thorny skate, *Raja radiata*, tagged in the Newfoundland area. *J. Northw. Atl. Fish. Sci*., **5**: 55–63.

WALKER, P. A., and J. R. G. HISLOP. 1998. Sensitive skates or resilient rays? Spatial and temporal shifts in ray species composition in the central and north-western North Sea between 1930 and the present day. *ICES J. Mar. Sci.*, **55**: 392–808.

WRIGHT, S. 1951. The genetic structure of populations. *Ann. Eugen.*, **15**: 323–354.

Other Useful References Suggested by the Authors

BUCKLEY, A. A., and J. D. METCALFE. 2002. The movement and behaviour of thornback ray, *Raja clavata*, in the Thames Estuary. 4th Meeting of the European Elasmobranch Association Proceedings, Paris, France Societe-francaise d'Ichtyologie, 191 p.

HOLDE, M. J. 1974. Ray migrations – do bigger eggs mean better dispersal. *Proc. Challenger. Soc*., **4**(5): 215 p.

WALKER, P., G. HOWLETT, and R. MILLNER. Distribution, movement and stock structure of three ray species in the North Sea and eastern English Channel. *ICES J. Mar. Sci*., Vol. 54(5): 797–808.

DUBAUT, M. H. 1977. Age and growth of *Raja batis* and *Raja naevus* in the Celtic Sea. *Jo. Cons. CIEM*, **37**(3): 261–265.