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Biochemical Evidence for a Third Species of Angel Shark off the East Coast of South America

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Key Word Index—*Squatina*; Squatinidae; Elasmobranch; electrophoresis; isoelectric focusing; isozymes; biochemical taxonomy.

Abstract—Two sibling species of angel shark (*Squatina* spp.) are known to exist off the coast of Brazil. Morphological evidence suggests that there may be a rare third species in the same area. The three putative species are compared using isozyme analysis and isoelectric focusing of sarcoplasmatic proteins. The loci *Est-1, Est-2, Est-3, Est-4* and *Sod-2* are diagnostic for the third species. The genetic identity of this species when compared with each of the other two species is 0.513. The results from isoelectric focusing of muscle proteins show typical patterns differing in each of the three species. The agreement between the morphological data, allozyme data and isoelectric focusing patterns indicates that the rare third putative species is biologically valid.

Introduction

Angel sharks (*Squatina* spp.) are of considerable economic importance in Southern Brazil and Argentina [1, 2]. Until recently, it was generally accepted that there was only one species, *S. argentina* (Marini, 1930) in the region [3–7] although some workers had previously suggested, on morphological grounds, that there might be two species [8–10]. Use of biochemical genetics to study gene pools in *Squatina* has recently shown that there are indeed two abundant species of this genus in the south of Brazil [11]. These occupy different, although overlapping, ranges of depth and can be clearly separated morphologically. Further morphological work [1] has recently suggested the presence of a third, less common, species in the same region. Here we provide evidence to confirm this suggestion, using the genetic analysis of isozyme patterns, a technique that has previously been extensively used in solving taxonomic problems in a variety of organisms [12–16]. Another technique used here is the analysis of isoelectric focusing patterns of sarcoplasmic proteins, a method well established for the identification of industrially processed fish products [17–19], but only comparatively rarely used in taxonomy [20].

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Results

Two individuals of *Squatina* morphotype III were found to have unique alleles at three of the 10 loci analysed. Additionally, the absence of enzymatic activity at the loci *Est-1* and *Est-2*, in fishes of morphotype III was interpreted as being due to the presence of fixed null alleles at those loci (null alleles have been previously described for esterases from other species of fish [21, 22]). The gene frequencies observed at the loci studied are presented in Table 1. The calculated genetic identity [23] and similarity [14] indices are, respectively, 0.712 and 0.673 between morphotypes I and II, and 0.513 and 0.500 between either of these and morphotype III. These values are within the range normally found in comparisons of congeneric species [14–16]. The isoelectric focusing patterns were also very different in each morphotype (Figs 2 and 3). The results obtained with the Coefficient of Racial Likeness (C.R.L.) index [24] and Coefficient of Distance (C.D.) index [25] for the comparisons of the morphotypes studied are shown in Table 2.

Discussion

Although the sample size of the third

TABLE 1. GENE FREQUENCIES FOR THE LOCI ANALYSED IN THE THREE MORPHOTYPES OF *SQUATINA ARGENTINA*

Loci	Alleles	Morphotypes I	II	III
AlP	100	1.00	1.00	1.00
Est-1	100	1.00	1.00	0.00
	null	0.00	0.00	1.00
Est-2	100	1.00	1.00	0.00
	null	0.00	0.00	1.00
Est-3	100	0.42	0.00	0.00
	89	0.58	0.00	0.00
	95	0.00	0.41	0.00
	86	0.00	0.00	1.00
	84	0.00	0.59	0.00
Est-4	100	0.00	1.00	0.00
	92	1.00	0.00	0.00
	80	0.00	0.00	1.00
Ldh	100	1.00	1.00	1.00
Mdh	100	1.00	1.00	1.00
Me	100	1.00	1.00	1.00
Sod-1	100	1.00	1.00	1.00
Sod-2	100	1.00	1.00	0.00
	60	0.00	0.00	1.00

Sample sizes are: morphotype I, 19; morphotype II, 29; morphotype III, 2.

morphotype is small, it is still adequate for the calculation of indices of genetic distance [26, 27]. In any case between populations which are sympatric, as in the present work, the presence of even one diagnostic locus is enough to demonstrate reproductive isolation [13]. Morphotype III presented five diagnostic loci in

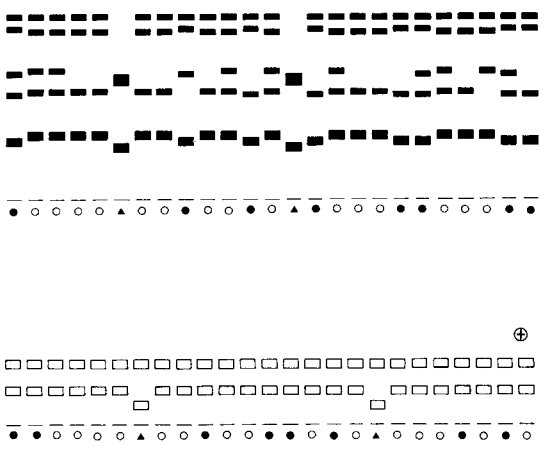

FIG. 1. DRAWINGS FROM TYPICAL POLYACRYLAMIDE GELS STAINED FOR EST AND SOD. *Est-1* and *Est-2* are assumed to be monomorphic for a null allele in morphotype III. Morphotype I (●); morphotype II (○); morphotype III (▲).

TABLE 2. COEFFICIENT OF RACIAL LIKENESS (C.R.L.) AND COEFFICIENT OF DISTANCE (C.D.) FROM PAIRWISE COMPARISONS OF THE THREE MORPHOTYPES OF *SQUATINA*, BASED ON THE MEAN RELATIVE AREAS OF ISOELECTRIC FOCUSING PATTERNS OF SARCOPLASMIC PROTEINS

	I and II	I and III	II and III
C.R.L.	9.72	4.56	4.54
C.D.	0.61	0.78	0.68

relation to morphotypes I and II. Such results would be very unlikely to be observed by chance if morphotype III is freely interbreeding with either of the other two morphotypes. For example, for any of these loci, the expected frequency of a new allele in morphotype I is lower than 1/38 [i.e. lower than 1/(2 × sample size)]. The expected frequency of a homozygote for this supposedly new allele in morphotype I would be lower than $(1/38)^2$. Therefore, the probability P that the two individuals of morphotype III actually belong to the same populations as morphotype I and present by chance differences in five loci simultaneously is lower than $\{[(1/38)^2]^2\}^5$ (i.e. $P<2.54 \times 10^{-32}$). Because of the larger sample size of morphotype II, the probability of simultaneous random differences at five loci in two individuals is even smaller ($P<\{[(1/58)^2]^2\}^5$ or $P<5.39 \times 10^{-36}$). Further discussion about this kind of approach to the analysis of small samples can be found in Carter and Thorpe [28].

Isoelectric focusing

The bands 2, 4, 7, 8, 11 and 13 are the most important in differentiating the three morphotypes (Fig. 2). However, bands 4, 11, and 13 alone are enough for each morphotype to be identified with confidence (Fig. 3). Due to the shortage in the literature of comparable data using area analysis of isoelectric focusing patterns in comparisons of intra- or interspecific populations, it is not possible to estimate the taxonomic significance of the calculated distance indices. However, it is clear that they agree with the enzyme data in indicating that morphotypes I and II are more closely related to each other than either is to morphotype III. Despite the difficulties in establishing the genetic coding of general proteins, isoelectric

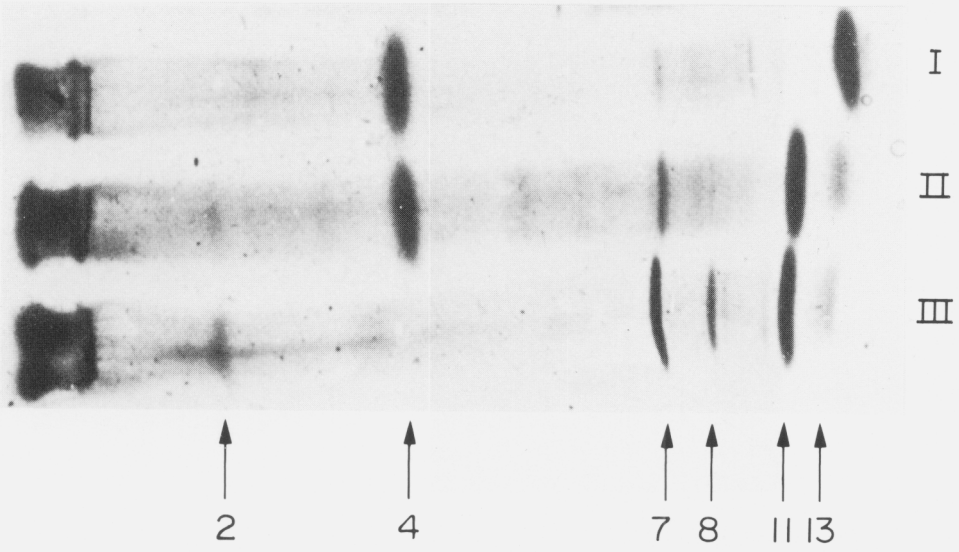

FIG. 2. TYPICAL ISOELECTRIC FOCUSING PATTERNS OF MUSCLE EXTRACTS FROM THE THREE MORPHOTYPES OF *SQUATINA ARGENTINA*.

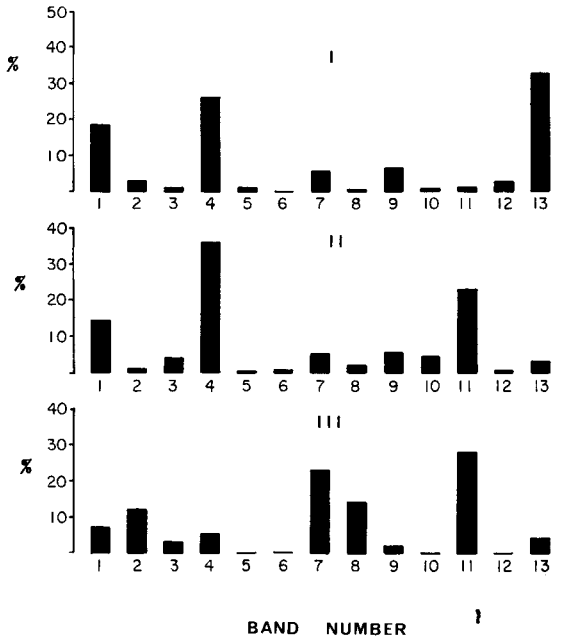

FIG. 3. HISTOGRAM OF MEANS OF RELATIVE AREAS OF THE 13 MOST IMPORTANT (RELATIVE AREA >1%) PEAKS FROM THE DENSITOGRAMS OF THE ISOELECTRICFOCUSING PATTERNS OF SARCOPLASMIC PROTEINS FROM THE THREE MORPHOTYPES OF *SQUATINA ARGENTINA* (SAMPLE SIZES AS IN TABLE 1).

focusing of total soluble proteins would appear to have potential as a tool for the estimation of overall distances between species. It has been previously shown that intraspecific variation of these patterns is usually very small [17, 29].

The coherence between the morphological and genetic data [1, 11], the interspecific differences in protein patterns and the consequent probability of the absence of gene flow must indicate that individuals of morphotype III belong to a different species from morphotypes I and II, although probably still of the genus *Squatina*. Morphotypes I and III have been described by Vooren [1] as *Squatina guggenheim* Marini, [9] and *Squatina argentina* Marini [8]. Morphotype II is probably a new species [1; Vooren, personal communication]. There is a clear need for further studies of populations of South American angel sharks over a wider geographical range. The identification of morphotype III as belonging to *S. argentina* (*sensu* Marini [8]) indicates that this species may be more abundant in areas further south than those from which samples were obtained during the present study. The recent discovery ([1, 11], this paper) of the three different biological species within the nominate species *Squatina argentina* suggests that the 12 recognized species within the genus [7] may well be an underestimate. As with the three species considered here, species differences are likely to be masked by high morphological similarity within this particularly conservative [30] and somewhat specialized [31] genus. A reappraisal of all the squatinid species worldwide and of their relationships to other elasmobranch families is probably overdue.

Experimental

Angel sharks were trawled in 1981 and 1982, during cruises of the R.V. *Atlantico Sul*. Collections were from 30 to 34 S off the coast of Brazil, at depths between 20 and 120 m. The classification of the individuals into morphotypes was carried out on board, using the morphological criteria given in Table 3. Morphotypes I and II were available in large numbers, but many trawls throughout the year over a wide geographical area produced very few individuals of morphotype III. Samples of heart, liver and white muscle were taken from 19 individuals of morphotype I, 29 individuals of morphotype II and 2 individuals of morphotype III. The tissues were always taken from freshly killed mature fish, and were immediately frozen at −20° until analysis. Electrophoresis, using homogenized samples, was on 7% polyacrylmide slab gels or on cellulose acetate plates (Chemetron, Milan). The conditions of extraction, electrophoresis of the samples, staining of the gels and nomenclature of isozyme loci are as previously described [11]. Enzymes studies were EST, SOD, LDH, MDH, ME and AIP, producing a total of 10 scorable loci. The genetic separation of morphotypes I (= *Squatina guggenheim* Marini [9]) and II (=*Squatina* sp.) has been established in earlier work [11]. Isoelectric focusing of muscle extracts was made using LK-plates, pH 3.5–10.0 (LKB, Broma, Sweden) run for 7 h at 4°, at a maximum power of 3 W, and maximum of 1000 V. Fixation, staining and preservation of the plates followed standard procedures [18, 29]. The stained plates were scanned at 540 nm in a single beam densitometer (Varian, UV-vis 634–5), and the relative areas of selected peaks calculated manually. To obtain a measure of

TABLE 3. MORPHOLOGICAL CHARACTERISTICS OF THE THREE MORPHOTYPES OF *S. ARGENTINA* (BASED ON [1] AND [11])

Characteristic	Morphotype I	II	III
Dorsal spines	present	absent	absent
Number of teeth in upper jaw	20–22	18–20	24–26
Maximum size (cm)	87	131	137
Number of functional ovaries	1	1	2
Length of pectoral fin/total length	0.31	0.32	0.37
Sample size	19	19	9

overall similarity between the species studied, the Pearson's Coefficient of Racial Likeness (C.R.L.) [24] and the Clark's Coefficient of Distance (C.D.) [25] were used. The two coefficients differ in that C.D. takes into account only the differences between the means of the relative areas of the densitogram peaks, whereas C.R.L. uses, in addition to those differences, the standard deviations as sources of information on similarity [32].

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