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Protein differences among the Mediterranean species of the genus *Spicara*

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Protein electrophoresis (PAGE) was used to study the three morphologically different species of *Spicara* (*S. flexuosa*, *S. maena*, *S. smaris*). Of the 28 enzymatic and additional myogenic loci, five monomorphic loci (*LDH-1**, *G6PD-1**, *PGI-1** and two *PMMs**) were species-specific markers of *S. smaris* with respect to *S. flexuosa* and *S. maena.* Four of the 28 enzymatic loci were polymorphic (*EST-1**, *GLDH**, *PEPD**, *PGI-2**). Discriminating genetic markers were not identified between *S. flexuosa* and *S. maena*. Genetic distance (*D*) as calculated by Nei's index (1978), between *S. smaris v. S. maena* and *S. flexuosa* showed a value, respectively of *D*=0·137 and 0·141. Between *S. flexuosa* and *S. maena* the value was *D*=0·006. From the data it can be inferred that *S. flexuosa* and *S. maena* are conspecific, despite morphological differences.
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Key words: protein electrophoresis; species differentiation; genetic distance; *Spicara*; Mediterranean Sea.

INTRODUCTION

The genus *Spicara* is common in shallow rocky and mud bottoms all around the Mediterranean, Black Sea, Portugal to Morocco and the Canary Islands (Tortonese, 1975, 1986). This genus has posed numerous identification problems and consequently many species have been described, leading to a variety of synonyms; this was attributed to marked variations in coloration related to the effects of sexual dichromatism (sex inversion, state of sexual maturity) (Zei, 1941; Lozano Cabo, 1951, 1953; Lepori, 1959). Many Mediterranean fishes show notable chromatic and morphological modifications especially in the juvenile phase and during the reproductive period (Tortonese, 1975).

The old classification distinguished the two genera *Maena* and *Smaris* that were subsequently fused in a single genus *Spicara* (Tortonese, 1975), which comprises three species: *Spicara flexuosa* (Rafinesque, 1810), *S. maena* (Linnaeus, 1758) and *S. smaris* (Linnaeus, 1758).

Currently, *Spicara smaris* is a very characteristic species, whereas *S. maena* may be distinguished from *S. flexuosa* owing to the presence in the former of well-developed teeth on the vomer, to its head being shorter than the body depth, to sexual behaviour, and to variations in coloration (Tortonese, 1975, 1986). According to Tortonese (1975), *S. flexuosa* should be considered as a colour polymorphism of *S. maena*. Pollard & Pichot (1971), in reviewing this genus

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through electrophoretic studies of densitometric eye-lens proteins and morphometric analysis, proposed the presence of only two species: *S. smaris* and *S. chryselis*. The latter is a synonym of *S. flexuosa* (Tortonese, 1975).

Since genetic differences between species can be evaluated through electrophoretic analysis of proteins (Altukhov, 1982; Ayala, 1983; Carvalho *et al.*, 1991), in the present research, electrophoretic analysis of several enzymes and myogens were carried out, and the genetic distance between *S. flexuosa*, *S. maena* and *S. smaris* was determined using Nei's (1978) index.

MATERIALS AND METHODS

SAMPLES

Samples of *S. flexuosa* (n=58), *S. maena* (n=55) and *S. smaris* (n=55) were obtained from the Gulf of Palermo, Sicily, Italy. The individuals were identified according to Tortonese (1975).

The frozen fishes were taken to the laboratory, and kept at − 20° C until used. Tissue from eye, heart, liver and muscle were homogenized in one volume of NaCl 1% at 0° C. The extracts were centrifuged at 6000 ***g*** at 4° C for 30 min, and the supernatant was paper-filtered to remove the lipid layer. When not used immediately the homogenates were stored at − 80° C.

ELECTROPHORETIC ANALYSIS

Polyacrylamide gel slab electrophoresis (PAGE) was carried out as described by Davis (1964). The sample, 1–5 μl sample-buffer, was deposited into each well of the spacer gel (16 × 16 cm, 2 mm thick) and run vertically at a constant current of 40 mA. When examined for myogen patterns (*PMM*) the gel was stained with Coomassie Brilliant Blue (Merril, 1990).

GEL STAINING FOR ISOENZYMES

Nomenclature for protein-coding loci and alleles followed the recommendations by Shaklee *et al.* (1990) (Table I). Running buffers and staining procedures were those previously reported by Richardson *et al.* (1986) and Cammarata *et al.* (1991).

Before staining, the gel slabs were incubated in the appropriate reaction mixture at 37° C until the bands were visualized; the reaction was stopped by rinsing with water and adding preservative solution (7% acetic acid).

STATISTICS

A locus was considered as polymorphic, when the frequency of the most common allele was <0·95 (Ayala, 1975). Genetic distance (*D*) was calculated from the formula proposed by Nei (1978) using the BIOSYS-1 program (Swofford & Selander, 1981). The obtained values were clustered in the UPGMA algorithm using the NTSYS (Rohlf, 1988) program which gives hierarchical levels according to genetic similarity.

RESULTS

Eighteen enzymes were resolved, and 28 loci scored in the three species (Table I). In addition, several loci encoding for myogens were examined.

Of the 28 enzymatic and additional myogenic loci, five monomorphic loci (*LDH-1**, *G6PD**, *PGI-1** and two *PMMs**) were species-specific between *Spicara smaris v. S. flexuosa* and *S. smaris v. S. maena*, whereas no discriminating monomorphic locus was identified between *S. flexuosa* and *S. maena*

TABLE I. Enzymes stained, with E.C. No., abbreviation and loci scored

Enzyme	E.C. No.	Abbreviation	Loci scored	Tissue
Alcohol dehydrogenase	1.1.1.1	ADH	*ADH**	L
Sorbitol dehydrogenase	1.1.1.14	SDH	*SDH**	L
Lactate dehydrogenase	1.1.1.27	LDH	*LDH-1,2,3**	E
Malate dehydrogenase	1.1.1.37	MDH	*MDH-1,2**	E
Glucose dehydrogenase	1.1.1.47	GLDH	*GLDH**	L
Glucose-6-phosphate dehydrogenase	1.1.1.49	G6PD	*G6PD**	H
Isocitrate dehydrogenase	1.1.1.42	IDH	*IDH**	L
Xanthine dehydrogenase	1.2.1.37	XDH	*XDH**	L
Xanthine oxidase	1.2.3.2	XO	*XO**	L
Superoxide dismutase	1.15.1.1	SOD	*SOD**	L, M
Aspartate aminotransferase	2.6.1.1	AAT	*AAT**	L
Adenylate Kinase	2.7.4.3	AK	*AK-1,2**	L
Phosphoglucomutase	2.7.5.1	PGM	*PGM-1,2**	M
Peptidase	3.4.– –	PEPB	*PEPB-1,2**	L
	3.4.13.9	PEPD	*PEPD**	L
Esterase	3.1.1.1	EST	*EST-1,2,3**	L
Fumarate hydratase	4.2.1.2	FUM	*FUM-1,2**	M
Glucose phosphate isomerase	5.3.1.9	PGI	*PGI-1,2**	E, L, M
General muscle proteins	na	PMM	*Several*	M

na: not applicable; E: eye; H: heart; L: liver; M: muscle.

(Table II) (Fig. 1). In particular, *LDH-1**, *G6PD-1** and *PGI-1** monomorphic loci were fixed with different alleles in these species. Allele **93* of the *LDH-1** locus was exclusive of *S. smaris* and allele **100* of the *LDH-1** locus was present in *S. flexuosa* and *S. maena*; allele **71* of the *G6PD-1** was found in *S. smaris* while allele **100* of the *G6PD-1** was observed in *S. flexuosa* and *S. maena*; allele **78* of the *PGI-1** locus was specific for *S. smaris*, and allele **100* of the *PGI-1** locus for *S. flexuosa* and *S. maena*. Two markers were present in the myogenic electrophoretic patterns. Of the 28 enzymatic loci scored, five were polymorphic; the polymorphic locus *EST-2** presented unclear patterns and was not considered further. In order to estimate the differences among genotypes of the three species (*S. smaris*, *S. flexuosa* and *S. maena*), the genetic distances (D) were calculated using Nei's (1978) index. The distance values between *S. smaris* and *S. flexuosa* (D=0·141) and between *S. smaris* and *S. maena* (D=0·137) were rather large, whereas the distance between *S. flexuosa* and *S. maena* were only D=0·006 (Fig. 2).

DISCUSSION

Although the genetic variation limited to protein encoding loci may lead to an underestimate of genetic diversity, our findings reveal that they were useful to discriminate among the *Spicara* species group. Allozyme and myogen diversity did not reflect the systematic relationships reported through analyses of morphological characters and species biology (Tortonese, 1975). *Spicara smaris* appeared to diverge from *S. flexuosa* and *S. maena* in the expression of five monomorphic loci of the 28 loci scored. The alleles of the *LDH-1**, *G6PD-1**,

Table II. Allele frequencies in *Spicara flexuosa*, *Spicara maena* and *Spicara smaris*

Loci	Alleles	*S. flexuosa*	*S. maena*	*S. smaris*
*ADH**	**100*	1	1	1
*SDH**	**100*	1	1	1
*LDH-1**	**93*	0	0	1
	**100*	1	1	0
*LDH-2**	**100*	1	1	1
*LDH-3**	**100*	1	1	1
*MDH-1**	**100*	1	1	1
*MDH-2**	**100*	1	1	1
*GLDH**	**95*	0	0·14	0·15
	**100*	0·36	0·23	0·77
	**135*	0·13	0·45	0·04
	**156*	0·51	0·18	0·04
*G6PD-1**	**71*	0	0	1
	**100*	1	1	0
*IDH**	**100*	1	1	1
*XDH**	**100*	1	1	1
*XO**	**100*	1	1	1
*SOD**	**100*	1	1	1
*AAT**	**100*	1	1	1
*AK-1**	**100*	1	1	1
*AK-2**	**100*	1	1	1
*PGM-1**	**100*	1	1	1
*PGM-2**	**100*	1	1	1
*PEPB-1**	**100*	1	1	1
*PEPB-2**	**100*	1	1	1
*PEPD**	**87*	0·21	0·29	0·05
	**100*	0·63	0·52	0·74
	**104*	0·16	0·19	0·21
*EST-1**	**83*	0·12	0·00	0·00
	**90*	0·03	0·15	0·07
	**92*	0·03	0·15	0·00
	**100*	0·56	0·58	0·61
	**110*	0·25	0·12	0·32
*EST-3**	**100*	1	1	1
*FUM-1**	**100*	1	1	1
*FUM-2**	**100*	1	1	1
*PGI-1**	**78*	0	0	1
	**100*	1	1	0
*PGI-2**	**94*	0·00	0·04	0·11
	**100*	0·97	0·96	0·89
	**109*	0·03	0·00	0·00

*PGI-1** monomorphic loci and two *PMMs**, were species-specific genetic markers for *S. smaris*. The level of genetic diversity expressed by Nei's index showed a value of D=0·141 for *S. flexuosa v. S. smaris* and D=0·137 for *S. maena v. S. smaris* which are in agreement with the values for congeneric fish species reported by Thorpe (1982). The genetic distance between *S. flexuosa v.*

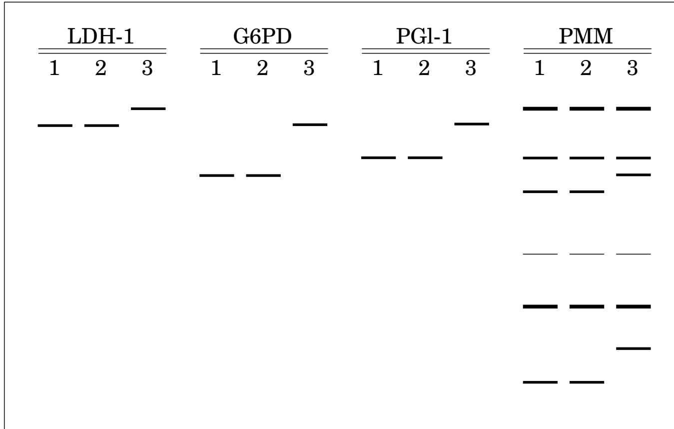

FIG. 1. Species-specific markers of monomorphic loci in the genus *Spicara*: 1, *S. maena*; 2, *S. flexuosa*; 3, *S. smaris*.

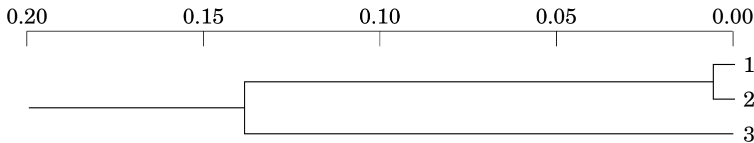

FIG. 2. UPGMA dendrogram based on Nei's (1978) index (*D*). 1, *Spicara flexuosa*; 2, *S. maena*; 3, *S. smaris*.

S. maena showed a value of $D=0{\cdot}006$ which parallels their morphological similarity.

According to Pollard & Pichot (1971), who studied the eye-lens proteins by densitometry and morphological characters, and Tortonese (1975), who described the weak discriminatory power of specific morphological characters (chromatic variability, presence or absence of teeth on the vomer), our results suggest that in the genus *Spicara*, *S. maena* and *S. flexuosa* can be distinguished easily from *S. smaris*. Therefore, it is necessary to investigate specifically the relationship between *S. flexuosa* and *S. maena* to verify the hypothesis of Pollard & Pichot (1971) and Tortonese (1975) who considered *S. flexuosa* to be a chromatic ecophenotype or habitat-coloration of *S. maena*: our data support such an hypothesis. Further research by means of additional protein loci and DNA analysis, will contribute to evaluate the degree of variability between *S. flexuosa* and *S. maena*.

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