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ECOSYSTEMS AND SUSTAINABILITY

Identification of subpopulations in pelagic marine fish species using amino acid composition

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Abstract The spatial stock complexity of marine fish species requires that population structure is taken into account in fisheries management. The aim of this study was to determine whether the amino acid composition (AAC) of the adult fish allows the identification of subpopulations within the stock. During a cruise in November 2003 along the entire Mediterranean coast of Spain, individuals were collected of the following pelagic species: *Sardina pilchardus*, *Sardinella aurita*, *Engraulis encrasicolus*, *Trachurus trachurus*, *Trachurus mediterraneus*, *Scomber scombrus* and *Scomber colias*. Individuals of *S. pilchardus* and *E. encrasicolus* were also collected from the waters of the Strait of Sicily in 2002 and 2003. The AAC of the fish eyes was seen to be species specific, and therefore, the differences in AAC among species may be based on inherited characters. Moreover, a clear differentiation was seen between the Spanish and Sicilian populations of *S. pilchardus* and *E. encrasicolus*. Furthermore, in the Spanish waters of the Mediterranean Sea, discriminant analysis revealed a substantial separation between the northern and southern subpopulations of *S. pilchardus*, *S. aurita* and *E. encrasicolus*. Temporal variations in AAC within species in each area were lower than the spatial variations observed

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among areas for each species, probably reflecting the influence on the AAC of the contrasting environmental characteristics of each area. Our results indicate that the ACC of the eyes in adult fish is a good tool for discriminating among subpopulations in pelagic marine fish species.

Keywords Pelagic fishes · Amino acid composition · Population discrimination

Introduction

Fisheries management is usually based upon stock units. A fish stock can be defined as a population adapted to a particular environment, having genetic differences from other stocks as a consequence of this adaptation (MacLean & Evans, 1981).

Fish stock discrimination has been measured in a number of ways (Pawson & Jennings, 1996; Cadrin et al., 2005). Some of these stock identification techniques have been questioned, as they reflect environmental distinctiveness rather than reproductive isolation (see Swain & Foote, 1999). Genetic studies often fail to support the stock differentiation suggested by morphologic, meristic, physiologic and/or ecologic variability (Ryman et al., 1984; Kinsey et al., 1994; Turan et al., 1998). However, these methods reveal that not all individuals within the stock are affected by the same environmental conditions and, therefore, failure to recognize or to account for the spatial complexity of subpopulations within a stock may lead to the erosion of subpopulation units, with unknown consequences on stock viability (Stephenson, 1999).

The recognition of this complex spatial structure within areas, traditionally assumed to contain a single stock, led to the 'dynamic population structure concept' (Smith & Jamieson, 1986), which suggests that most fish species exist as semi-independent but not completely isolated breeding units. This concept gained support among biologists against that of a pure or discrete stock (Spanakis et al., 1989). This dynamic population model is the equivalent to considering the dynamic of marine fish populations from a metapopulation perspective (MacQuinn, 1997; Bailey et al., 1999; Stephenson, 1999; Thorrold et al., 2001; Smedbol & Wroblewski, 2002).

Andrewartha & Birch (1954) suggested that populations are structured in 'local populations' connected by migration. Levins (1970) developed this concept by coining the term metapopulation to describe a population consisting of many local populations.

In some marine fish species, such as herring (MacQuinn, 1997) and sardine (Carrera & Porteiro, 2003), a new perspective of metapopulation, different from the classical concept of Levins (1970), has been proposed to explain population dynamics. Metapopulation is a system of discrete local populations each of which, to a large extent, determines its own internal dynamics, but with a degree of identifiable and nontrivial demographic influence from other local populations through the dispersal of individuals, according to the definition of Kritzer & Sale (2004). These authors put less emphasis on the extinction dynamics and defined metapopulation according to spatial structure (and the role that interpopulation exchange plays in local population size and stability).

A necessary condition for metapopulation persistence in the face of unstable subpopulations is asynchronous local dynamics (Hanski, 1999), which means that subpopulations may experience different environmental conditions. This indicates that the factors structuring habitat suitability may vary among subpopulations. Therefore, for a correct stock management it is necessary to identify subpopulations within the stock that are affected by different environmental conditions, and to manage these as discrete groups.

The problem is to find a methodology that allows the discrimination of subpopulations within the stock (Waldman, 1999) and, for that reason, some ecologists may remain unimpressed by the metapopulation concept (Hanski, 1999). Fish subpopulations are difficult to define from traditional fisheries data, or to discriminate by conventional stock identification techniques (Stephenson, 1999). The methodology must be based on inherited characters, which also must reflect environmental distinctiveness and must be species specific (Booke, 1999). To discriminate stocks or subpopulations of fishes, the signal from among-stock variation must exceed the noise of within-stock variation (Waldman, 1999).

The amino acid composition (AAC) of the eggs and larvae of marine fishes have been successfully used to discriminate among species and spawning

areas within species (Riveiro et al., 2003). One of the main problems of the study of biochemical composition is the finding of correct part of the fish body to analyse, as many fish change their biochemical composition rapidly, depending on the characteristics of the geographic area.

The aim of this study was to determine whether the AAC of some of the tissues of adult fish allows the identification of subpopulations in several pelagic fish species in the Mediterranean Sea.

Materials and methods

Sampling collection

To determine the best part of the fish body for discriminating among species, individuals of the species *Sardina pilchardus* and *Engraulis encrasicolus* were collected in the Sicilian Channel on-board fishing vessels in March 2002 (Fig. 1). The liver, blood, heart and eyes of males and females were extracted from the fishes and transferred to ultracentrifuge plastic vials and immediately frozen at −32°C. The number of individuals analysed is shown in Table 1.

For the identification of subpopulations, individuals of the species *Sardina pilchardus*, *Trachurus trachurus*, *Trachurus mediterraneus*, *Scomber scombrus*, *Scomber colias*, *Engraulis encrasicolus* and *Sardinella aurita* were collected in November–December 2003 during the ECOMED survey along the Spanish Mediterranean coast (Fig. 1, Table 1). Two areas were considered for collecting samples: the northern and southern areas separated by San Antonio Cape. Both areas have been shown to be potentially favourable habitats for fish reproduction, but with contrasting conditions (Agostini & Bakun, 2002).

Individuals of the species *E. encrasicolus* and *S. pilchardus* were collected in December 2003, again on-board fishing vessels in the same areas as in the previous sampling in the Sicilian Channel (Fig. 1, Table 1). The eyes of males and females were extracted from the fishes and transferred to ultracentrifuge plastic vials and immediately frozen at −32°C. The number of individuals analysed by species and group origins is shown in Table 1.

Analyses of amino acids

Total amino acids were analysed by high-performance liquid chromatography (HPLC) using a Waters Alliance System, a Waters 474 scanning fluorescence detector and a Waters 15 X 3.9 Nova-Pack C18 column following the method described by Van Wandelen & Cohen (1997). The AAC was analysed using the Waters AccQ-Tag® Chemistry Package. For the hydrolysis procedure for AAC, eyes were introduced in HCl 6 N at 114°C for 24 h. Afterwards, vials were introduced in a desiccator with NaOH at 55°C in order to dry the samples. For the derivatization procedure,

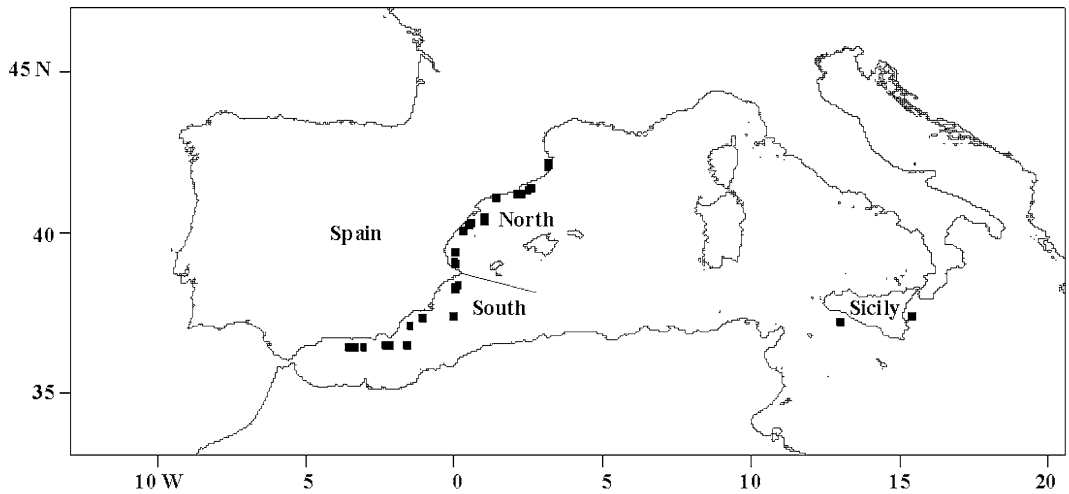

Fig. 1 Mediterranean Sea divided in arbitrary areas for this study and the sampling locations of each area (*squares*)

Table 1 Number of individuals collected for the analysis of the AAC of the different fish tissues in Sicily and Spanish Mediterranean Coast in 2002 and 2003

Year:	2002				2003		
Tissue:	Blood	Heart	Liver	Eye	Eye		
Area:	Sicily				Spain		Sicily
					North	South	
Sardina pilchardus	15	10	9	10	45	21	4
Engraulis encrasicolus	26	19	19	20	47	27	
Sardinella aurita					49	25	12
Trachurus trachurus					30	17	
Trachurus mediterraneus					12	17	
Scomber scombrus					8		
Scomber colias					22		

samples were redissolved in HCl 0.1 N and ACCQ-Fluor® Borate Buffer and AccQ-Fluor® Reagent were added and samples were placed for 10 min at 55°C. Conditions for amino acid separation for the mobile phase were as follows: A (Aqueous buffer, AccQ-Tag Eluent), B (acetonitrile), C (bidistilled water). A total of 15 amino acids were analysed: aspartic acid (ASP), serine (SER), glutamic acid (GLU), glycine (GLY), histidine (HIS), arginine (ARG), threonine (THR), alanine (ALA), proline (PRO), tyrosine (TYR), valine (VAL), lysine (LYS), isoleucine (ILE), leucine (LEU) and phenylalanine (PHE). Cysteine and methionine were destroyed during hydrolysis with 6 N HCl while amino acid standard H NCI0180 Pierce H was used for the identification and quantification of amino acids.

Discrimination among subpopulations

To determine whether it is possible to discriminate among subpopulations within species according to the AAC of the adult tissues, a standard multivariate discriminant analysis was applied to the amino acid percentages (Guisande et al., 2006). The variables that contribute most to sample differentiation were identified by their correlation to the discriminant functions and by a one-way ANOVA. The resultant discriminant functions were used to classify the individuals into samples, and the percentage of individuals correctly classified into the original sample was used to evaluate the classification success rate. All calculations were performed using SPSS software (SPSS Inc.).

Discriminant analysis has become a powerful tool in biological research and stock identification and has been successfully used to discriminate among species, stocks or spawning areas in pelagic fishes according to the AAC of the larvae (Riveiro et al., 2003; Cuttitta et al., 2006), elemental composition in fish otoliths (Geffen et al., 2003), fish parasites (Timi et al., 2005; Marques et al., 2006), etc.

Results

Table 2 shows the results of the discriminant analysis applied to the AAC of the different parts of the body

Table 2 Results of a discriminant analysis showing the percentage of individuals correctly classified according to their AAC

	Real group	Predicted groups	
		S. pilchardus	*E. encrasicolus*
Blood (97.4%)	*S. pilchardus*	100	0
	E. encrasicolus	3.8	96.2
Heart (100%)	*S. pilchardus*	100	0
	E. encrasicolus	0	100
Liver (100%)	*S. pilchardus*	100	0
	E. encrasicolus	0	100
Eye (100%)	*S. pilchardus*	100	0
	E. encrasicolus	0	100

of the adult fish from the Sicilian Channel in March 2002. It can be seen that the AAC of the blood was not the best fish tissue for discriminating among species, as the percentage of individuals correctly classified for the sardine and the anchovy was not 100%. Nevertheless, the AAC of the liver, heart an eyes were good indicators of the species, as they allowed a perfect separation of the sardine and the anchovy (Table 2).

Following these preliminary results, it was decided that only fish eyes would be sampled, as this was the easiest way to obtain samples without having to dissect fish.

A discriminant analysis performed on the data of the AAC of eyes collected in 2003 of *S. pilchardus* (*S.p.*), *S. aurita* (*S.a.*), *E. encrasicolus* (*E.e.*), *T. trachurus* (*T.t.*), *T. mediterraneus* (*T.m.*), *S. scombrus* (*S.s.*) and *S. colias* (*S.c.*) from the Sicilian and Spanish coasts, showed that it is possible to identify each fish species according to their AAC (Fig. 2). The significant functions were from 1 to 5 (Wilk's lambda, $P < 0.005$). The first two components of the discriminant analysis explained 62.4 and 29.1% of variance, respectively (Fig. 3). The percentage of fish correctly classified according to their AAC represented 73.7% of cases with cross validation (Fig. 3). Among the different species studied, the major similarities were found between *T. trachurus* and *T. mediterraneus* and between *S. pilchardus* and *S. aurita* (Fig. 3). The AAC of eyes mainly differs among species in the proportion of histidine, glycine and valine.

In a discriminant analysis performed on the AAC of the eyes of adults collected in 2003 from different areas of the Mediterranean coast of Spain (north and south) and from the Sicilian coast (only *S. pilchardus* and *E. encrasicolus*) (Table 3), the percentage of groups correctly classified was 64.3% for *S. pilchardus*, 75.7% for *S. aurita*, 74.4% for *E. encrasicolus*, 85% for *T. trachurus* and 86.9% for *T. mediterraneus* (Fig. 4). It was not possible to perform discriminant analysis for *S. scombrus* and *S. colias*, because the samples of these species were only found in the northern area of the Mediterranean coast of Spain. The AAC of eyes mainly differs among species in the proportion of glycine, proline and threonine for *S. pilchardus*, serine, threonine and arginine for *S. aurita*, tyrosine, serine and proline for *E. encrasicolus*, glycine, isoleucine and proline for *T. trachurus* and tyrosine, serine and histidine for *T. mediterraneus.*

The use of AAC allows discriminating among species and among areas within species: between the species of the Sicilian and Spanish coasts (*S. pilchardus* and *E. encrasicolus*), and also between those of the northern and southern areas of the Mediterranean Sea (Fig. 4). It is important to point out that the results of the discrimination between the Spanish and Sicilian coasts have to be interpreted with caution, because of the differences in sample

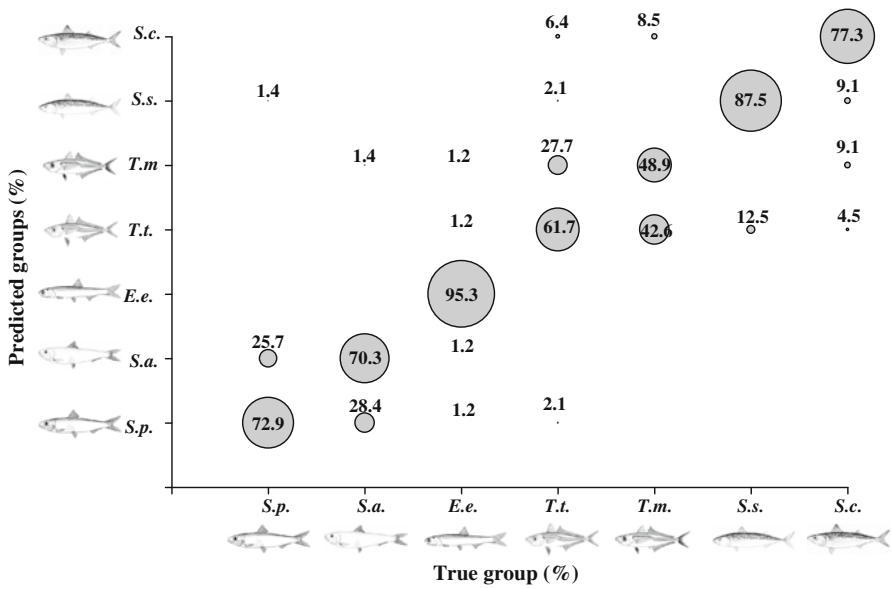

Fig. 2 Plot of the results of a discriminant analysis showing the percentage of fish species correctly classified from the original data according to the amino acid composition of the eyes. Species abbreviations as shown in Materials and Methods

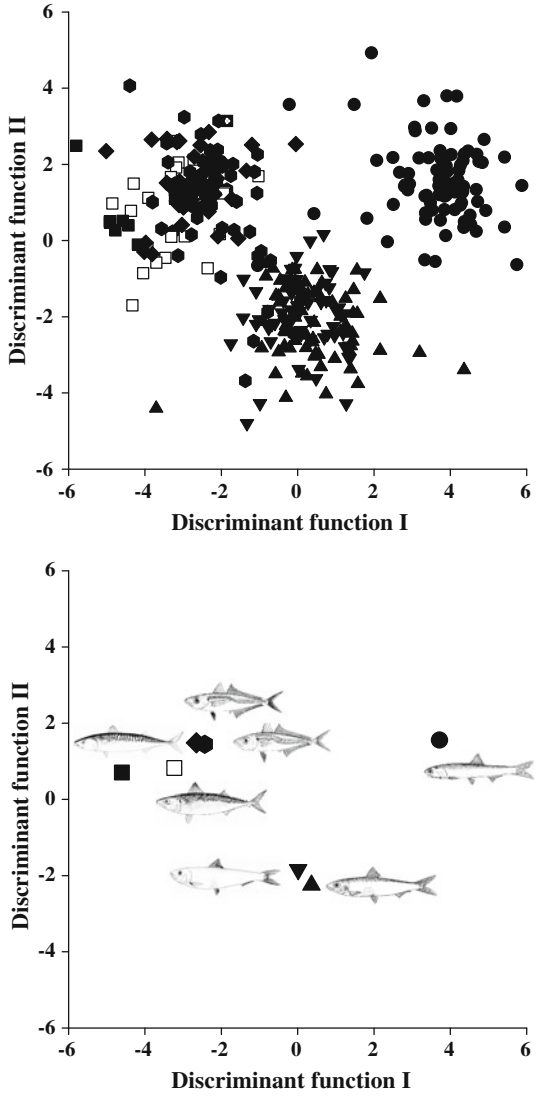

Fig. 3 Plot of the first two discriminant function scores obtained from the discriminant analysis performed on the amino acid composition of fish eyes. *Sardina pilchardus* (*triangle*), *Sardinella aurita* (*inverted triangle*), *Engraulis encrasicolus* (*circle*), *Trachurus trachurus* (*hexagon*), *Trachurus mediterraneus* (*diamond*), *Scomber scombrus* (*filled square*), *Scomber colias* (*open square*). **a** Plot of the individuals. **b** Plot of the species centroids

size between the two areas, especially given the small amount of data from the Sicilian coast (Table 1).

Furthermore, it was necessary to test whether there is temporal variation in the AAC within species in the same area. A discriminant analysis was performed on the AAC of the eyes of adult fish collected in 2003 for the different distribution areas of the Mediterranean coast of Spain (north and south) and for *S. pilchardus* and *E. encrasicolus* on the Sicilian coast. However, for Sicily, data from individuals collected in 2002 (without assigned group) were also included. Eventually, all data from Sicily in 2002 were assigned to the group for Sicily in 2003 (Table 4).

Discussion

The starting point for using the AAC for stock separation applications is that the habitat characteristics from separate geographical areas could influence AAC for fish living in those areas. Although, to our knowledge, it had never been used before, our results reveal that the AAC of the eyes provides a method for practical discrimination of fish subpopulations, where the amount of exchanges between subpopulations is low, but sufficient to prevent genetic differentiation (see Lleonart & Maynou, 2003). Therefore, this method is useful for studying the fine-scale population structure of marine fishes.

Our results show that it is possible to discriminate pelagic fish species and fish distribution based on the AAC of the eyes. A good differentiation was seen between the Spanish and Sicilian populations of *S. pilchardus* and *E. encrasicolus,* which concur with previous studies that have shown the existence of several subpopulations of both species in the Mediterranean Sea (Larrañeta, 1968; Spanakis et al., 1989; Bembo et al., 1996a, b; Tudela, 1999; Borsa, 2002). These observed differences could be a result of the contrasting environmental conditions experienced by fish in the studied areas (Agostini & Bakun, 2002).

In the Spanish Mediterranean Sea, discriminant analysis also produced a good separation between northern and southern subpopulations of *S. pilchardus*, *S. aurita* and *E. encrasicolus*. Differences in AAC are in agreement with genetic differences (Ramon & Castro, 1997), with differences in growth rates (Alemany & Alvárez, 1993) and with differences in biometric, physiologic and fisheries characteristics (Larrañeta, 1968), reported for northern and southern subpopulations of *S. pilchardus.*

Genetic techniques cannot detect differences when there are low levels of larval or adult mixing between subpopulations (Edmonds et al., 1989; Hartl & Clark, 1989; Swan et al., 2006) and, therefore, are not useful

Table 3 Amino acid composition (mean ± SD weight percentage of yields of total amino acids) of fish eyes

	S. pilchardus			*S. aurita*		*E encrasicolus*			*T. trachurus*		*T. mediterraneus*		*S. scomber*	*S. colias*
	Spain N	Spain S	Sicily	Spain N	Spain S	Spain N	Spain S	Sicily	Spain N	Spain S	Spain N	Spain S	Spain N	Spain N
ASP	11.3 ± 0.9	11.3 ± 1.5	10.8 ± 0.5	11.5 ± 1.6	12.6 ± 1.8	11.1 ± 13	12.2 ± 1.5	10.3 ± 0.9	11.4 ± 1.5	11.6 ± 1.7	11.9 ± 1.6	11.3 ± 1.7	10.8 ± 1.0	11.8 ± 1.6
SER	5.7 ± 0.6	6.1 ± 0.9	6.2 ± 0.9	6.1 ± 0.8	5.5 ± 0.5	5.7 ± 0.9	5.2 ± 0.6	6.6 ± 0.8	6.3 ± 0.8	6.3 ± 0.8	5.9 ± 1.0	6.7 ± 0.8	6.9 ± 0.8	6.0 ± 0.7
GLU	14.4 ± 3.9	12.4 ± 3.6	10.4 ± 3.0	12.8 ± 3.4	14.7 ± 3.6	13.3 ± 3.6	15.5 ± 3.8	10.3 ± 2.3	14.0 ± 3.8	15.1 ± 3.8	13.8 ± 3.3	12.9 ± 3.7	12.3 ± 3.7	15.2 ± 3.6
GLY	10.0 ± 0.9	10.0 ± 0.7	11.8 ± 1.4	10.1 ± 1.0	9.6 ± 0.7	9.2 ± 0.7	8.4 ± 0.5	9.9 ± 0.7	10.4 ± 0.9	9.1 ± 1.0	9.8 ± 0.8	9.9 ± 1.0	12.3 ± 0.5	11.2 ± 1.0
HIS	3.4 ± 0.4	3.4 ± 0.2	3.0 ± 0.3	3.3 ± 0.4	3.3 ± 0.3	4.3 ± 0.3	3.9 ± 0.3	4.1 ± 0.4	3.4 ± 0.3	3.2 ± 0.3	3.4 ± 0.2	3.2 ± 0.4	3.3 ± 0.2	3.7 ± 0.6
ARG	5.7 ± 0.4	5.9 ± 0.5	5.7 ± 0.6	6.3 ± 0.6	5.8 ± 0.7	6.7 ± 0.6	6.2 ± 0.6	6.6 ± 0.5	7.0 ± 0.8	6.6 ± 0.8	6.9 ± 1.0	6.8 ± 0.9	6.2 ± 1.0	5.9 ± 1.0
THR	4.5 ± 0.4	4.6 ± 0.4	5.0 ± 0.8	4.6 ± 0.4	4.2 ± 0.4	4.4 ± 0.3	4.2 ± 0.9	4.3 ± 0.5	4.8 ± 0.4	4.6 ± 0.6	4.8 ± 0.4	4.8 ± 0.5	4.7 ± 0.3	4.7 ± 0.5
ALA	6.3 ± 0.8	6.5 ± 0.8	6.7 ± 1.3	6.0 ± 0.7	6.0 ± 0.4	5.7 ± 0.5	5.9 ± 0.8	6.1 ± 1.1	5.6 ± 0.5	5.8 ± 1.9	5.6 ± 0.7	6.1 ± 0.8	6.3 ± 0.4	5.6 ± 0.8
PRO	5.2 ± 0.4	5.3 ± 0.4	5.8 ± 0.6	5.2 ± 0.6	5.0 ± 0.4	4.9 ± 0.4	4.9 ± 0.4	5.4 ± 0.4	5.0 ± 0.4	4.6 ± 0.5	4.9 ± 0.4	5.2 ± 0.4	5.8 ± 0.3	5.0 ± 0.7
TYR	6.6 ± 0.8	6.6 ± 0.8	6.4 ± 1.7	6.8 ± 0.8	6.4 ± 0.7	7.6 ± 0.7	6.8 ± 0.8	9.4 ± 1.5	7.1 ± 0.8	6.5 ± 0.8	7.0 ± 0.6	5.9 ± 0.5	6.5 ± 0.6	7.2 ± 1.4
VAL	5.5 ± 0.7	5.8 ± 0.6	6.0 ± 0.5	5.7 ± 0.5	5.7 ± 0.4	5.7 ± 0.5	5.8 ± 0.4	5.4 ± 0.6	4.9 ± 0.5	5.4 ± 0.8	5.2 ± 0.5	5.5 ± 0.5	4.7 ± 0.3	4.4 ± 0.6
LYS	5.4 ± 0.7	5.6 ± 0.8	5.1 ± 0.7	5.2 ± 0.9	5.4 ± 0.4	5.0 ± 0.6	5.2 ± 0.9	4.6 ± 0.6	4.8 ± 0.6	5.4 ± 1.8	4.8 ± 0.8	5.3 ± 0.7	5.0 ± 0.4	4.4 ± 1.0
ILE	3.8 ± 0.5	4.0 ± 0.4	4.1 ± 0.2	4.0 ± 0.4	4.0 ± 0.3	4.3 ± 0.5	4.3 ± 0.3	3.9 ± 0.5	3.7 ± 0.4	4.3 ± 0.6	4.1 ± 0.5	4.3 ± 0.4	3.6 ± 0.3	3.3 ± 0.6
LEU	6.3 ± 0.7	6.5 ± 0.6	6.9 ± 1.0	6.3 ± 0.6	6.1 ± 0.5	6.0 ± 0.5	5.9 ± 0.6	6.1 ± 0.6	5.9 ± 0.5	6.0 ± 0.9	6.1 ± 0.5	6.4 ± 0.6	6.1 ± 0.2	5.7 ± 0.7
PHE	5.9 ± 0.6	6.1 ± 0.5	6.1 ± 0.8	6.0 ± 0.7	5.8 ± 0.6	6.2 ± 0.6	5.7 ± 0.6	7.0 ± 0.6	5.6 ± 0.5	5.6 ± 0.9	5.8 ± 0.5	5.5 ± 0.5	5.5 ± 0.4	5.8 ± 1.1

Amino acid abbreviations as in text

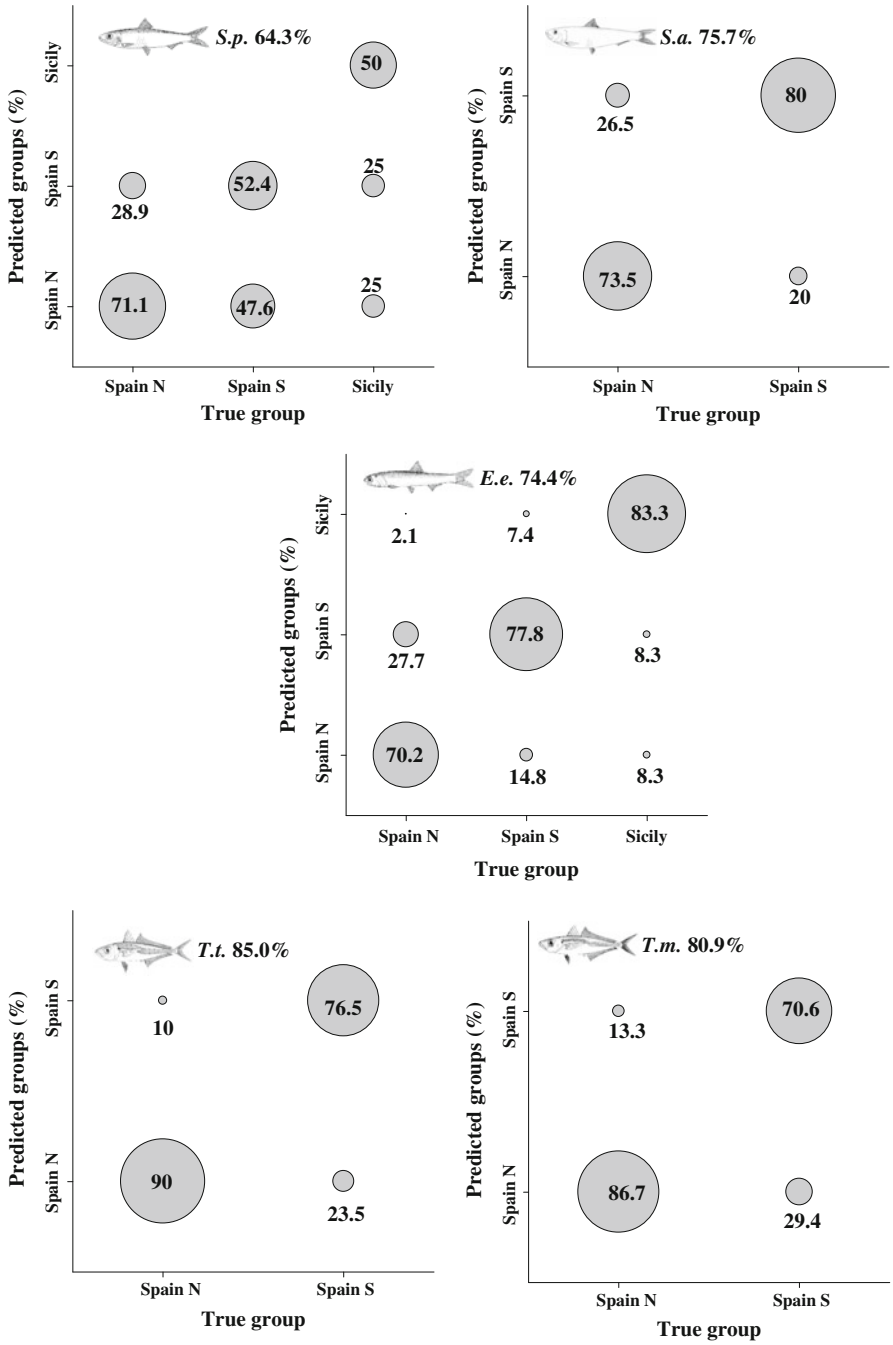

Fig. 4 Plot of the results of a discriminant analysis showing the percentage of fish species from different areas correctly classified from the original data according to the amino acid composition of the eyes. Species abbreviations as shown in Materials and Methods

for resolving the fine-scale population structure of marine fishes (to discriminate among subpopulations). Relatively low levels of exchange between stocks, negligible from a management perspective, may be sufficient to ensure genetic homogeneity (Ward & Grewe, 1994). Moreover, even if a larger number of loci are used, because the percentage of DNA expressed as proteins is low compared to total DNA available, it is uncertain whether the DNA sequence analysed is important in terms of the adaptation of the species to the habitat (Kocher, 2003). Hence, although stock discrimination derived from DNA sequences accurately represents the history of genes, it does not necessarily reflect the

Table 4 Classification results of the discriminant analysis showing percentage of cases assigned to each group for *S. pilchardus* and *E. encrasicolus*

	Predicted group		
	Spain N (%)	Spain S (%)	Sicily (%)
Real group			
S. pilchardus			
Spain N	77.8	22.2	0
Spain S	19.0	81.0	0
Sicily 2003	0	0	100
Sicily 2002 (without assigned group)	0	0	100
E. encrasicolus			
Spain N	78.7	24.3	0
Spain S	7.4	88.9	3.7
Sicily 2003	0	0	100
Sicily 2002 (without assigned group)	0	0	100

history of the population in which the variants are found.

Differences in habitat conditions, due to adaptation to different photic environments (spectral composition of shallow bodies of water is highly variable due largely to differences in the quantity and identity of the substances dissolved), may have consequences upon eye pigments, tuned by the AAC within the opsin (the protein that determines the spectral absorption characteristics of the light) (see Douglas et al., 1998).

Other methods, such as the elemental composition of otoliths, have been used as a means for resolving the fine-scale stock and population structures of marine fishes (Thresher, 1999; Thorrold et al., 2001; Geffen et al., 2003; Rooker et al., 2003; Swan et al., 2006). One of the problems of otolith chemistry is that it shows strong year-to-year variability and must be used as a seasonally stable biological tracer (Campana et al., 2000), as nursery fingerprints have been shown to vary between years (Gillanders et al., 2001; Gillanders, 2002).

Although intensive sampling is necessary for all species and areas over a long period (more than 3–5 years), our preliminary results comparing different sampling years for *S. pilchardus* and *E. encrasicolus* revealed that AAC may be used as a stable tag in pelagic fishes.

It is not suggested that the definition of the population structure of marine fishes should rely upon this amino acid technique. There is still a need for a holistic (multiple technique) approach for the study of the population structure of marine fishes (Waldman et al., 1997; Begg & Waldman, 1999; Murta, 2000), as different methods may produce different results. The information obtained from the analysis of amino acids would be complementary information to that obtained from other techniques.

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