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Electrophoretic analysis of stock structure in Northern Mediterranean anchovies, *Engraulis encrasicolus*

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Starch gel electrophoresis of allozymes was used to investigate the genetic stock structure of European anchovies (*Engraulis encrasicolus* L.) in the northern Mediterranean area. Twenty-four putative enzyme-coding loci were examined in 634 fish, constituting 13 samples caught in the Adriatic, Tyrrhenian, Ionian, and Aegean seas, the Sicilian Channel and the Bay of Biscay, between March 1993 and May 1994. Eight loci were monomorphic in all samples, eight were weakly polymorphic ($p=0.99$), and a further eight exhibited common allele frequencies <0.95 ($p=0.95$). Over 99% of tests indicated that genotypic proportions were in accordance with Hardy-Weinberg predictions. Average mean unbiased heterogeneity per locus (H_L) was 0.055 and the effective mean number of alleles per locus was 1.17. Genetic distance between samples was low (maximum Nei's $D=0.009$).

An outgroup sample from the Bay of Biscay was easily distinguishable from Mediterranean fish, due to fixation at a number of polymorphic loci. Chi-square analyses revealed significant differences ($p<0.05$) between Mediterranean samples at six loci (*G3PDH-1**, *G3PDH-2**, *IDHP-2**, *mMEP**, *GAPDH-1** and *GPI**), and *GAPDH-1** and *GPI** in particular showed obvious heterogeneity between seas. Gene diversity analysis indicated that 96.6% of variation was within samples, with only 3.4% between samples (G_{ST}). 77% of this between-sample variation was partitioned between seas, the remainder accounting for spatial and temporal variation within seas.

The results are discussed in relation to the hydrographic and physical barriers to migration in the study area, and compared with those obtained by other workers. The possible consequences of stock integrity in clupeoid fish with regard to resilience and recovery from collapse are discussed.
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Introduction

Since the 1970s, starch gel electrophoresis of proteins has been used to assay genetic variation within and between populations of many marine teleosts (see, for example, Smith and Fujio (1982)). The usual intention is to investigate the genetic integrity of stocks targeted by commercial fisheries, although the lack of barriers to migration and mixing in the ocean environment compared with freshwater generally results in little intraspecific genetic divergence, even with considerable geographic distance. Gyllensten (1985) commented that total genetic variation (gene diversity H_T) and intra-population diversity (G_S) may be higher in marine species, probably due to large population sizes and high migration rates, but that the component of diversity allocated between geographic locations (G_{ST}) was lower for these taxa. However, given the economic and social value of sustained marine fisheries, the potential benefits of stock discrimination and associated management strategies result in the application of genetic methods to an ever-increasing list of species (Carvalho and Hauser, 1994; Ward and Grewe, 1994).

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Clupeoid fisheries present several problems for management. Low density-dependence in their stock-recruitment relationship makes them susceptible to overfishing (Grahame, 1987), and mixed fisheries often exist where the biomass of two or more similar species vary inversely (Gulland and Garcia, 1984). Effective stock discrimination would greatly aid management in such situations; hence, considerable efforts have been made to elucidate genetic stock structure in these fishes, albeit with mixed success. Using allozyme analysis, Grant and Utter (1984) distinguished two races of Pacific herring (*Clupea pallasi* Val.) in the Asian–Bering Sea and eastern North Pacific, and also differentiated a number of spawning locations within these areas. Kornfield *et al.* (1982) detected a degree of allozyme variation between Atlantic herring (*Clupea harengus harengus* L.) spawning grounds in the Gulf of Maine, but found it to be temporally unstable, noting that temporal differences between Autumn and Spring spawners were greater in magnitude than any spatial patterns. Minimal polymorphism was detected in oil sardine *Sardinella longiceps* Val. samples from the Indian coastline (Menezes, 1994), and in the absence of any genetic sub-structuring it was concluded that they had been collected from a single panmictic population.

The engraulids, growing to 10–20 cm, are typically coastal schooling planktivores with batch spawning. The European anchovy *Engraulis encrasicolus* L. is found along the Eastern Atlantic coastline from Norway to Angola in West Africa, and in the Mediterranean, Black, and Azov seas, where it is a principal target species for commercial fisheries. Indeed, the annual catch in the Mediterranean and associated seas reached almost 700 000 t in the early 1980s (Whitehead *et al.*, 1988), and, as an example, constitutes around 25% of the Italian pelagic catch (Bombace, 1992). Several workers have reported population differences within and between Black and Azov Sea anchovies (Altukhov and Salmenkova, 1981; Kalnin *et al.*, 1984; Kalnina and Kalnin, 1984), but, considering the value of European anchovy fisheries, there have been relatively few studies on the relationships and interactions between stocks in the open seas around the Mediterranean. A notable exception was the work of Spanakis *et al.* (1989) who recognised that the Aegean and Ionian Sea populations were not panmictic, although their results indicated that a degree of gene flow occurred between these areas. Recently, Garcia *et al.* (1994) examined *E. encrasicolus* from the north-west Mediterranean, but were unable to differentiate between samples from sites covering an area from Barcelona on the Spanish coast to close to the island of Elba on the Western Italian coast.

In this paper we examine the genetic stock structure of the European anchovy in the northern Mediterranean. Our samples are taken from within and around the Adriatic Sea, some of the most productive waters in the Mediterranean, and also extend into the Tyrrhenian, Ionian, and Aegean seas, facilitating comparisons with published data.

Materials and methods

Anchovies (n=40–50 per sample) were taken by commercial fishing vessels in the Mediterranean and immediately placed on dry ice. On return to port, samples were kept at −80°C until required. Figure 1 shows sampling locations for the Mediterranean. The Bay of Biscay outgroup sample was captured in March 1993 at 49°10′N, 5°30′W, approximately midway between Brest and Plymouth. These fish were stored at −30°C for four months before electrophoresis.

Muscle, eye, and liver tissues were dissected from each fish, and screening was carried out to optimize conditions for the activity and resolution of allozymes using standard methods of horizontal starch gel electrophoresis (May, 1992). Tissues were disrupted in 15–75 µl 0.01 M Tris-HCl buffer, pH 7.5, using a Teflon homogenizer (a small amount of sterile sand was also added to muscle samples). Samples were centrifuged at 3000 *g* for 3 min in a microfuge, and the supernatant blotted with filter paper wicks. A total of 34 enzyme stains and six buffer systems (Appendix 1) were assayed using 13% hydrolysed starch (Connaught Laboratories). Ferritin was used as a standard to facilitate comparisons of mobility across gels, although all alleles at each locus were also run on a single gel in order to check relative mobilities.

Nomenclature for enzyme loci and allele designation followed the recommendations of Shaklee *et al.* (1990). Alleles were denoted according to their mobility relative to the most commonly observed allele, which was assigned a mobility of 100 units. Within-sample variation was assessed by calculating mean unbiased expected heterozygosity per locus, H_L (Nei, 1978), mean number of alleles per locus, mean effective number of alleles per locus (the reciprocal of the sum of squares of allele frequencies), the percentage of loci [(i) exhibiting any degree of polymorphism ($p=0.99$), and (ii) those with common allele frequencies <0.95 ($p=0.95$)]. Agreement with Hardy-Weinberg expectations of genotype frequency was tested by chi-squared statistics, and by testing the significance of Wright's fixation index, F_{is} (Brown, 1970). Allele frequencies were also compared between samples (and between subsets of samples) by chi-squared testing. Tables of probability values were tested for the random occurrence of significant statistics by a sequential Bonferroni procedure (Rice, 1989), which eliminates type I errors. Genetic distance between samples was calculated as Nei's D (1972) and Cavalli-Svorza and Edwards' (1967) chord distance. Partitioning of genetic variation across polymorphic loci was accomplished by gene diversity analysis (Nei, 1973;

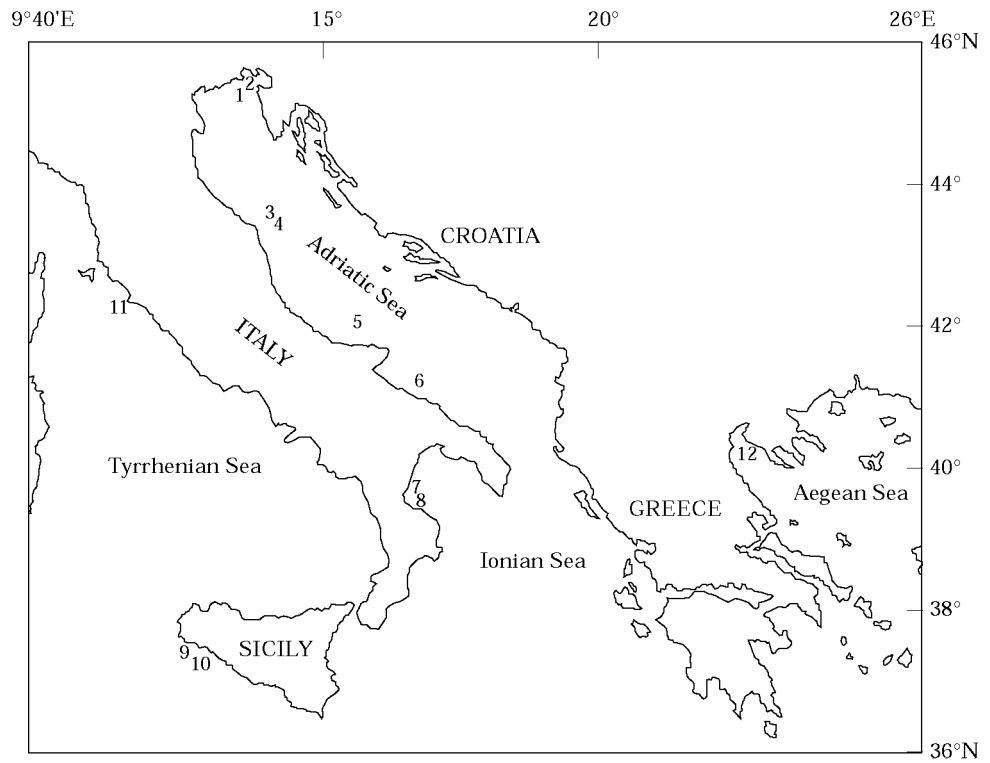

Figure 1. Map of the northern Mediterranean showing sample sites where *E. encrasicolus* were collected. Sample month+year are given in parentheses. 1: Trieste (5/93), 2: Trieste (9/93), 3: Ancona (5/93), 4: Ancona (9/93), 5: Vieste (4/93), 6: Vieste (9/93), 7: Ionian Sea (5/93), 8: Ionian Sea (7/93), 9: Sicily (4/93), 10: Sicily (7/93), 11: Tyrrhenian Sea (2/94), 12: Aegean Sea (5/94).

Chakraborty, 1980). Genetic data were analysed using BIOSYS v1.7 software (Swofford & Selander, 1981), PHYLIP v3.5c (Felsenstein, 1993) and spreadsheet macros written for Lotus 1-2-3.

Results

15 enzyme stains encoded by 24 putative gene loci showed acceptable activity and resolution, and were chosen for routine sample analysis (Table 1), using Tris-maleic pH 8.9 and citrate morpholine pH 7.4 buffers (Carvalho & Loney, 1989). A total of 634 fish were analysed. Electrophoretic patterns were, in general, reasonably clear and interpretation was straightforward; electromorph patterns were in agreement with predictions of quaternary structure based on studies of other fish species (May, 1992). Storage of the Bay of Biscay samples at −30°C did not compromise electrophoretic analysis.

Of the 24 loci assayed routinely, eight (*CK-1**, *CK-3**, *ESTD**, *FH**, *GAPDH-2**, *GAPDH-3**, *MPI**, and *PGDH**) were monomorphic in all samples. A further eight loci (*CK-2**, *G6PDH**, *IDHP-1**, *LDH-2**, *MDH-1**, *MDH-2**, *MDH-3**, and *PGM**) were weakly polymorphic, with common allele frequencies >0.95 in all samples, while eight loci (*G3PDH-1**, *G3PDH-2**, *GAPDH-1**, *IDHP-2**, *LDH-1**, *mMEP**, *PEP**, and *PGM**) showed common allele frequencies <0.95 in at least one sample. Mean unbiased heterozygosity per locus H_L (Nei, 1978) (Table 2) varied from a minimum of 0.020 in the Biscay sample to 0.089 in the Aegean, with an average value of 0.055. The mean number of alleles per locus ranged from 1.25 (Biscay) to 1.67 (Aegean), with an average of 1.52. The effective number of alleles per locus showed a similar pattern within a lower range, Biscay fish again having the lowest value (1.03). The overall mean figure was 1.17. Bay of Biscay fish had the lowest number of polymorphic loci, regardless of whether p=0.99 or p=0.95, while the highest values were found in Mediterranean samples from outside the Adriatic Sea. Overall, 37% of loci were polymorphic with no criterion and 17% were polymorphic at p=0.95 (Table 2).

One out of 115 tests (<1%) of genotypic fit with Hardy-Weinberg predictions was significant using a chi-squared test corrected for small sample size (Levene, 1949) (*GPI**, Aegean Sea, $\chi^2=8.7$, df=3, p=0.033), and also by testing the significance of Wright's F_{is} ($p<0.05$).

Table 1. Enzymes routinely scored in *E. encrasicolus*, loci identified, optimum buffer system and source tissue. Buffer recipes are given in Appendix 1.

Enzyme	Loci	Buffer	Tissue
Creatine kinase (2.7.3.2)	*CK-1**	Cm	Eye
	*CK-2**		
	*CK-3**		
Esterase-D (3.1.1.1)	*ESTD**	TM	Liver
Fumarate hydratase (4.2.1.2)	*FH**	CM	Muscle
Glycerol-3-phosphate dehydrogenase (1.1.1.8)	*G3PDH-1**	TM	Muscle
	*G3PDH-2**	TM	Liver
Glucose-6-phosphate dehydrogenase (1.1.1.49)	*G6PDH**	TM	Eye
Glucose-6-phosphate isomerase (5.3.1.9)	*GPI**	TM	Muscle
Glyceraldehyde-3-phosphate dehydrogenase (1.2.1.12)	*GAPDH-1**	CM	Muscle
	*GAPDH-2**		
	*GAPDH-3**		
Isocitrate dehydrogenase (1.1.1.42)	*IDHP-1**	CM	Eye
	*IDHP-2**		
Lactate dehydrogenase (1.1.1.27)	*LDH-1**	CM	Eye
	*LDH-2**		
Malate dehydrogenase (1.1.1.37)	*MDH-1**	CM	Eye
	*MDH-2**		
	*MDH-3**		
Mannose phosphate isomerase (5.3.1.8)	*MPI**	TM	Muscle
Malic enzyme (1.1.1.40)	*mMEP**	CM	Eye
Peptidase (3.4.11–13)	*PEP**	CM	Muscle
Phosphogluconate dehydrogenase (1.1.1.44)	*PGDH**	TM	Eye
Phosphoglucose mutase (5.4.2.2)	*PGM**	TM	Eye

TM=Tris-maleic, pH 8.9; CM=citrate morpholine, pH 7.4.

Table 2. Measures of allozyme variability in anchovy samples. % loci polymorphic (p=0.95) are those which exhibit common allele frequencies <0.95.

Sample	Heterozygosity per locus (mean unbiased)	Mean number of alleles per locus	Mean effective number of alleles (N_e)	% loci polymorphic (no criterion)	% loci polymorphic (p=0.95)
Trieste 5/93	0.059	1.50	1.27	33.3	16.7
Trieste 9/93	0.059	1.54	1.24	41.7	20.8
Ancona 5/93	0.049	1.58	1.11	37.5	16.7
Ancona 9/93	0.048	1.50	1.35	33.3	12.5
Vieste 4/93	0.041	1.38	1.10	25.0	12.5
Vieste 9/93	0.049	1.50	1.13	37.5	12.5
Ionian 5/93	0.055	1.63	1.12	45.8	20.8
Ionian 7/93	0.041	1.42	1.09	37.5	8.3
Sicily 4/93	0.075	1.50	1.20	37.5	25.0
Sicily 7/93	0.078	1.63	1.21	45.8	25.0
Tyrrhenian	0.055	1.63	1.12	47.8	16.7
Aegean	0.089	1.67	1.29	37.5	33.3
Bay of Biscay	0.020	1.25	1.03	20.8	4.17
Mean (s.d.)	0.055 (0.017)	1.52 (0.11)	1.17 (0.31)	37.0 (7.5)	17.3 (7.4)

This result was rejected by sequential Bonferroni analysis, hence genotypes at all *E. encrasicolus* loci were in Hardy-Weinberg equilibrium.

Chi-squared analysis indicated that allele frequencies differed between the 13 samples for eight of the 16 polymorphic loci (*G3PDH-1**, *G3PDH-2**, *LDH-1**, *IDHP-2**, *mMEP**, *GAPDH-1**, *G6PDH**, and *GPI**) and also when all loci were considered together (Table 3). Sequential Bonferroni testing rejected the significant values for *mMEP** and *G6PDH**. Omitting the Bay of Biscay outgroup reduced the number of individual loci with significant chi-squared results to three after Bonferroni testing (*GAPDH-1**, *GPI**, and *IDHP-2**), but the test statistic was again significant when all loci were considered (Table 3). The change in significance at the *LDH-1** locus was due to the fact

Table 3. Chi-squared analysis comparing allele frequencies at polymorphic in anchovy samples. *p<0.05; **p<0.01; ***p<0.001.

	(i) All 13 samples, including Bay of Biscay				(ii) 12 Mediterranean samples only			
Locus	No. of alleles	χ^2	df	p	No. of alleles	χ^2	df	p
*CK-2**	2	11.7	12	0.4710	2	10.9	11	0.4526
*GAPDH-1**	2	199.9	12	<0.001***	2	184.4	11	<0.001***
*G3PDH-1**	3	46.7	24	0.0035**	3	39.2	22	0.0135*
*G3PDH-2**	5	91.8	48	<0.001***	4	53.1	33	0.0148*
*G6PDH**	2	21.6	12	0.0422*	2	19.3	11	0.0559
*GPI**	3	138.3	24	<0.001***	3	131.9	22	<0.001***
*IDHP-1**	2	18.2	12	0.1104	2	16.8	11	0.1126
*IDHP-2**	5	139.3	48	<0.001***	5	129.9	44	<0.001***
*LDH-1**	3	48.6	24	0.0021**	3	25.0	22	0.2950
*LDH-2**	2	11.7	12	0.4709	2	10.9	11	0.4526
*MDH-1**	2	11.7	12	0.4710	2	10.9	11	0.4526
*MDH-2**	3	21.4	24	0.6158	3	19.8	22	0.5964
*MDH-3**	3	23.3	24	0.4976	3	21.8	22	0.4733
*mMEP**	3	37.0	24	0.0439*	3	35.0	22	0.0390*
*PEP**	3	26.9	24	0.3078	3	23.8	22	0.3554
*PGM**	3	26.2	24	0.3434	3	23.6	22	0.3690
Total		874.5	360	<0.001***		756.3	319	<0.001***

that, although highly variable in the Mediterranean (*LDH-1*100*, 0.70–0.86), *LDH-1*100* was fixed in the Bay of Biscay sample (Table 4).

There was thus significant genetic heterogeneity between the Mediterranean samples, notably at the *IDHP-2**, *GPI**, and *GAPDH-1** loci (Table 4). *IDHP-2*100* frequencies ranged from 0.52 at Trieste in the North Adriatic to 0.88 in the Tyrrhenian Sea. *GPI** was only weakly polymorphic within the Adriatic (*GPI*100*, 0.97–1.00), but *GPI*100* frequencies fell as low as 0.80 (Sicily 4/93) in other areas. Similarly, the *GAPDH-1*100* allele was fixed in Adriatic samples but a *GAPDH-1*50* allele was found in all other areas of the Mediterranean.

Chi-squared comparisons of samples taken by vessels from the same ports at different times revealed no temporal differences between samples at Trieste (no individual loci significant; overall $\chi^2=11.4$, df=16, p=0.785), Ancona (no individual loci significant; overall $\chi^2=21.4$, df=16, p=0.161), Vieste (no individual loci significant; overall $\chi^2=15.6$, df=14, p=0.336), or Sicily (no individual loci significant; overall $\chi^2=15.6$, df=14, p=0.841). Samples from the Ionian Sea differed at *IDHP-2** ($\chi^2=8.7$, df=3, p=0.034) and *GPI** ($\chi^2=10.5$, df=1, p=0.001), although the value for *IDHP-2** was rejected by Bonferroni testing. Ionian Sea samples also differed significantly when all loci were considered together ($\chi^2=39.3$, df=17, p=0.001).

Calculated values of Nei's (1972) genetic distance (D) (Table 5) were extremely low for any pairwise comparison of samples (maximum value=0.009), precluding the construction of any informative dendrogram. Cavalli-Sforza and Edwards' (1967) chord distance varied from 0.038 (between the two Sicilian Channel samples) to 0.129 (between the Bay of Biscay and Aegean Sea fish), although the maximum value between any pair of Mediterranean samples was 0.108 (Trieste 9/93 and Aegean Sea). Figure 2 shows a consensus UPGMA dendrogram, based on 200 replicate datasets created by bootstrap resampling (Felsenstein, 1993). The consensus nodes (or branch points) shown occurred in 12–76% of individual trees (data not shown), values typical of such analyses in datasets where most of the genetic variability (and dendrogram structure) is associated with a small number of loci. In these cases, any bootstrapped dataset which omitted one of the key loci would result in a modified tree, hence reducing the frequencies of nodes appearing in the consensus dendrogram. The Bay of Biscay sample appears as an outlier, although its degree of separation reflects the limited genetic distance between all of the samples. Of the Mediterranean samples, those from the Adriatic group closely, with one Ionian sample located in this cluster.

Total gene diversity, H_T, was 0.092 when averaged across all loci (Table 6), while G_{ST}, the coefficient attributable to between-sample differentiation, was 0.034 (3.4%). G_{ST} was significantly different from zero ($t_s=3.052$, df=5, p=0.015), and was partitioned into diversity between seas (G_{SEA}), diversity between sampling ports within seas (G_{PS}, in this case, for the Adriatic Sea only) and diversity between temporal samples within ports (G_{SP}), i.e. $G_{ST}=G_{SEA}+G_{PS}+G_{SP}$. Over all loci, G_{SEA} made up 76.9% of between-sample variation (G_{SEA}/G_{ST}), G_{PS} 8.3% (G_{PS}/G_{ST}), and G_{SP} 14.8% (G_{SP}/G_{ST}), i.e. most of the between-sample variation was partitioned between seas, and not on a finer geographic or temporal scale. There were exceptions to this trend,

Table 4. Allele frequencies at polymorphic loci in samples of *E. encrasicolus*.

Locus	Allele	Sample												
		Trieste 05/93	Trieste 09/93	Ancona 05/93	Ancona 09/93	Vieste 04/93	Vieste 09/93	Ionian 05/93	Ionian 07/93	Sicily 04/93	Sicily 07/93	Tyrrhenian 02/94	Aegean 5/94	Biscay 3/93
*CK-2**	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00
	110	—	—	—	—	—	—	—	—	—	—	0.01	—	—
n		50	50	50	50	50	50	50	50	50	50	50	44	40
*G3PDH-1**	100	0.93	0.96	0.93	0.96	0.97	1.00	0.97	0.95	0.98	0.95	0.80	0.92	1.00
	77	0.07	0.04	0.06	0.04	0.03	—	0.03	0.05	0.02	0.05	0.20	0.08	—
	120	—	—	0.01	—	—	—	—	—	—	—	—	—	—
n		49	49	50	46	50	50	48	32	26	43	30	30	39
*G3PDH-2**	100	0.86	0.94	0.91	0.90	0.91	0.95	0.92	0.97	0.79	0.76	0.94	0.90	0.98
	120	0.02	0.01	0.01	—	—	—	0.01	—	0.01	0.03	0.01	0.02	—
	85	0.11	0.05	0.08	0.10	0.09	0.05	0.07	0.03	0.20	0.21	0.05	0.08	—
	65	0.01	—	—	—	—	—	—	—	—	—	—	—	—
	90	—	—	—	—	—	—	—	—	—	—	—	—	0.02
n		50	47	47	47	41	49	48	48	38	40	47	29	40
*G6PDH**	100	1.00	0.95	0.98	0.99	1.00	0.95	0.98	1.00	1.00	0.99	0.98	1.00	1.00
	85	—	0.05	0.02	0.01	—	—	0.02	—	—	0.01	0.02	—	—
	120	—	—	—	—	—	0.05	—	—	—	—	—	—	—
n		50	50	50	50	50	47	50	50	50	50	50	44	40
*GAPDH-1**	100	1.00	1.00	1.00	1.00	1.00	1.00	0.97	0.98	0.90	0.93	0.97	0.69	1.00
	50	—	—	—	—	—	—	0.03	0.02	0.10	0.07	0.03	0.031	—
n		50	50	50	50	50	50	50	50	50	50	50	44	40
*GPI**	100	0.97	1.00	0.99	0.97	1.00	1.00	0.90	1.00	0.80	0.81	1.00	0.85	0.97
	130	0.03	—	0.01	0.01	—	—	0.10	—	0.20	0.18	—	0.14	0.03
	50	—	—	—	0.02	—	—	—	—	—	0.01	—	0.01	—
n		50	50	50	50	50	50	50	50	50	50	50	44	39
*IDHP-1**	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	0.98	1.00	1.00
	200	—	—	—	—	—	—	—	0.01	—	—	0.02	—	—
n		50	50	50	50	50	50	50	50	50	50	50	44	40
*IDHP-2**	100	0.52	0.54	0.73	0.76	0.82	0.78	0.83	0.77	0.79	0.82	0.88	0.62	0.81
	125	0.47	0.42	0.21	0.22	0.17	0.17	0.11	0.21	0.18	0.14	0.06	0.35	0.13
	75	0.01	0.04	0.05	0.02	0.01	0.05	0.05	—	0.03	0.04	0.04	0.02	0.06
	160	—	—	0.01	—	—	—	0.01	0.02	—	—	0.02	—	—
	140	—	—	—	—	—	—	—	—	—	—	—	0.01	—
n		50	47	50	50	50	50	50	50	50	50	50	44	39

*LDH-1**	100	0.76	0.76	0.84	0.78	0.77	0.70	0.78	0.86	0.76	0.71	0.78	0.81	1.00
	140	0.24	0.24	0.16	0.22	0.23	0.30	0.22	0.14	0.24	0.29	0.22	0.18	—
	65	—	—	—	—	—	—	—	—	—	—	—	0.01	—
n		50	49	50	50	50	48	50	46	50	49	50	44	40
*LDH-2**	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00	0.98	1.00	1.00
	90	—	—	—	—	—	—	—	—	0.01	—	0.02	—	—
n		50	50	50	50	50	50	50	50	50	50	50	44	40
*MDH-1**	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00
	50	—	—	—	—	—	—	—	—	—	—	0.01	—	—
n		50	50	50	50	50	50	50	50	50	50	50	44	40
*MDH-2**	100	0.99	0.99	1.00	1.00	1.00	1.00	0.99	1.00	1.00	0.99	1.00	1.00	1.00
	50	—	0.01	—	—	—	—	0.01	—	—	0.01	—	—	—
	60	0.01	—	—	—	—	—	—	—	—	—	—	—	—
n		50	50	50	50	50	50	50	50	50	50	50	44	40
*MDH-3**	100	1.00	0.99	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	75	—	—	—	—	—	0.01	—	—	—	—	—	—	—
	125	—	0.01	—	—	—	—	—	—	—	—	—	—	—
n		50	50	50	50	50	50	50	50	50	50	50	44	40
*mMEP**	100	0.99	0.98	0.99	1.00	0.97	0.97	0.99	0.97	1.00	0.98	0.98	0.92	0.99
	115	0.01	0.02	—	—	0.02	0.01	—	0.03	—	0.02	—	0.03	0.01
	80	—	—	0.01	—	0.01	0.02	0.01	—	—	—	0.02	0.05	—
n		50	50	50	50	50	50	50	50	50	50	50	44	39
*PEP**	100	0.97	0.94	0.96	0.98	1.00	0.97	0.94	0.96	0.94	0.94	0.96	0.94	0.99
	70	0.02	0.03	0.01	0.01	—	0.01	0.01	0.04	0.04	0.02	0.02	0.05	0.01
	140	0.01	0.03	0.03	0.01	—	0.02	0.05	—	0.02	0.04	0.02	0.01	—
n		50	50	50	50	50	50	50	50	50	50	50	44	40
*PGM**	100	1.00	0.98	0.99	0.98	0.98	0.98	0.99	0.99	0.97	0.98	1.00	0.99	1.00
	135	—	0.02	—	0.01	0.01	0.02	—	—	—	—	—	—	—
	70	—	—	0.01	0.01	0.01	—	0.01	0.01	0.03	0.02	—	0.01	—
n		50	50	50	50	50	50	50	50	50	50	50	44	40

Table 5. Genetic distance between anchovy samples. Below diagonal: Cavalli-Sforza & Edwards' (1967) chord distance; above diagonal: Nei's (1972) genetic distance.

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13
1 Trieste 05/93	—	0.001	0.003	0.003	0.004	0.004	0.006	0.004	0.006	0.006	0.008	0.006	0.008
2 Trieste 09/93	0.055	—	0.002	0.002	0.003	0.003	0.004	0.003	0.006	0.007	0.007	0.006	0.006
3 Ancona 05/93	0.059	0.056	—	0.000	0.001	0.001	0.001	0.000	0.003	0.003	0.002	0.006	0.002
4 Ancona 09/93	0.056	0.055	0.040	—	0.000	0.001	0.001	0.001	0.003	0.003	0.002	0.006	0.003
5 Vieste 04/93	0.065	0.066	0.053	0.041	—	0.000	0.000	0.001	0.003	0.003	0.002	0.007	0.003
6 Vieste 09/93	0.063	0.058	0.042	0.044	0.047	—	0.001	0.001	0.003	0.003	0.002	0.007	0.004
7 Ionian Sea 05/93	0.077	0.078	0.047	0.058	0.071	0.050	—	0.001	0.002	0.002	0.002	0.006	0.003
8 Ionian Sea 07/93	0.069	0.072	0.062	0.058	0.051	0.066	0.076	—	0.004	0.004	0.002	0.006	0.001
9 Sicily 04/93	0.082	0.100	0.079	0.076	0.090	0.082	0.054	0.091	—	0.000	0.005	0.005	0.006
10 Sicily 07/93	0.081	0.093	0.076	0.073	0.087	0.075	0.048	0.093	0.038	—	0.004	0.006	0.007
11 Tyrrhenian Sea 02/94	0.091	0.087	0.060	0.072	0.075	0.066	0.067	0.071	0.098	0.091	—	0.009	0.004
12 Aegean Sea 05/94	0.094	0.108	0.099	0.101	0.087	0.100	0.085	0.096	0.072	0.075	0.106	—	0.009
13 Bay of Biscay 05/93	0.107	0.104	0.088	0.092	0.090	0.093	0.097	0.084	0.117	0.122	0.112	0.129	—

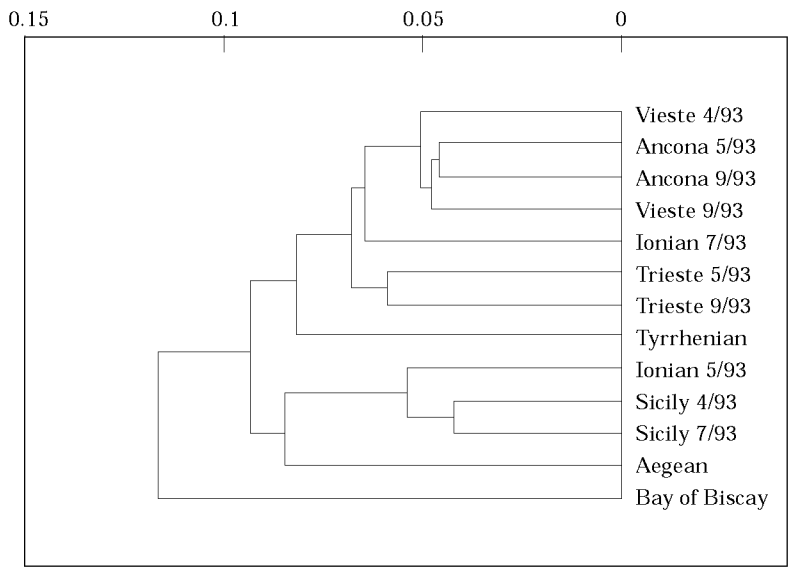

Figure 2. UPGMA dendrogram based on Cavalli-Sforza and Edwards' (1967) chord distance, showing the relationship between *E. encrasicolus* samples.

for example *PEP** and *G6PDH**, where G_{SEA} made up 28.5% and 18.3% of G_{ST}, respectively.

Discussion

Genetic variability

The levels of variation and electrophoretic mobilities of individual alleles found in our study of *E. encrasicolus* are broadly comparable with those of other workers, albeit with some exceptions. The range of allele frequencies in Kalnin *et al.* (1984) and Garcia *et al.* (1994) are, for common loci, similar to those of the current study, although nomenclature may differ as the numbering of loci obviously depends on the total number detected. We discount differences at, for example, *PEP**, where a variety of dipeptide substrates may result in the realization of different loci. One anomaly which is difficult to explain is variation at the *PGM** locus reported in Ionian and Aegean Sea samples by Spanakis *et al.* (1989), who quoted *PGM*100* frequencies of 0.63–0.91. In the same areas, *PGM*100* frequencies in our samples were all 0.99, and the range over all areas was 0.97–1.00 (Table 4), similar to values given by Garcia *et al.* (1994). In our study, resolution at this locus was clear and migration was sufficient that alleles with even small mobility differences should have been detected. Spanakis *et al.* used a modified Ridgway's buffer system (Ridgway *et al.*, 1970) for all of their enzyme systems, and it is feasible that technical differences in electrophoretic analysis may have resulted in the different scores obtained for this locus.

Smith and Fujio (1982) attempted to classify marine teleosts as habitat specialists or generalists, and commented on the trends in genetic variability within each group. Their values for observed heterozygosity in six species of Clupeiformes ranged from 0.058 in *Clupea pallasi* to 0.106 in *C. harengus*, the average figure being 0.074 (and the average for all marine teleosts being calculated as 0.055; Smith and Fujio, 1982). Our mean expected heterozygosity estimate of 0.058 (12 Mediterranean samples only) is at the bottom of the range for Clupeiformes, although three values exceeded 0.074 and a maximum value of 0.089 was recorded for the Aegean Sea sample. It is noteworthy that our values for expected heterozygosities were almost identical to observed values, as genotypes were in the Hardy-Weinberg equilibrium. The heterozygosity value of 0.020 in the Bay of Biscay sample is notably low, this sample being fixed for the common allele at loci which were usually polymorphic, e.g. *G3PDH-1**, *LDH-1** (Table 4). It may be that the relatively restricted size of the Biscay anchovy population (when compared with that in the Mediterranean), in conjunction with the large scale fluctuations in numbers characteristic of clupeoid fisheries, has resulted in a reduction in genetic diversity by the loss of rare alleles through stochastic processes. This unusually low index of variability justifies further analysis of the genetics of anchovy stocks in this area.

Deviation from Hardy-Weinberg predictions

Genotypic proportions differed significantly from Hardy-Weinberg expectations in one test only, less than

Table 6. Gene diversity analysis (Nei, 1973) of Mediterranean *E. encrasicolus* samples.

Variation	Locus						
	*G3PDH-1**	*G3PDH-2**	*LDH-1**	*LDH-2**	*IDHP-1**	*IDHP-2**	*PEP**
(a) Gene diversity (D)							
Total (H_T)	0.11275	0.18879	0.34796	0.00166	0.00499	0.42544	0.08339
Within samples (H_S)	0.10854	0.18218	0.34412	0.00165	0.00491	0.38399	0.08255
Between samples (D_{ST})	0.00421	0.00661	0.00384	0.00001	0.00007	0.04145	0.00083
Between seas (S_{SEA})	0.00389	0.00571	0.00143	6.9×10^{-6}	0.00006	0.01835	0.00024
Between ports within seas (D_{PS})	0.00011	0.00015	0.00098	0	0	0.01320	0.00016
Between samples within ports (D_{SP})	0.00021	0.00075	0.00143	8.3×10^{-6}	8.3×10^{-6}	0.00991	0.00044
(b) Coefficients of gene differentiation (G)							
Within samples (G_S)	0.96265	0.96498	0.98896	0.99082	0.98580	0.90256	0.98998
Between samples (G_{ST})	0.03735	0.03502	0.01104	0.00917	0.01420	0.09744	0.01002
Between seas (G_{SEA})	0.03453	0.03027	0.00410	0.00417	0.01253	0.04313	0.00286
Between ports within seas (G_{PS})	0.00095	0.00079	0.00282	0	0	0.03103	0.00186
Between samples within ports (G_{SP})	0.00186	0.00396	0.00411	0.00500	0.00167	0.02329	0.00530

Variation	Locus									
	*mMEP**	*MDH-1**	*MDH-2**	*MDH-3**	*G6PDH**	*GPI**	*PGM**	*GAPDH-1**	*CK-2**	Mean of all loci
(a) Gene diversity (D)										
Total (H_T)	0.04268	0.00166	0.00665	0.00499	0.02469	0.11125	0.02491	0.08852	0.00166	0.09200
Within samples (H_S)	0.04190	0.00165	0.00660	0.00495	0.02428	0.10032	0.02471	0.07427	0.00165	0.08677
Between samples (D_{ST})	0.00073	0.00001	0.00005	0.00004	0.00040	0.01092	0.00020	0.01424	0.00001	0.00523
Between seas (D_{SEA})	0.00051	0.00001	6.9×10^{-6}	9.7×10^{-6}	0.00007	0.00995	0.00011	0.01417	0.00001	0.00341
Between ports within seas (D_{PS})	0.00010	0	0.00002	5.6×10^{-6}	0.00004	5.6×10^{-6}	0.00001	0	0	0.00092
Between samples within ports (D_{SP})	0.00012	0	0.00002	0.00002	0.00029	0.00097	0.00007	0.00008	0	0.00089
(b) Coefficients of gene differentiation (G)										
Within samples (G_S)	0.98296	0.99083	0.99269	0.99193	0.98363	0.90180	0.99203	0.83904	0.99083	0.96572
Between samples (G_{ST})	0.01704	0.00917	0.00731	0.00808	0.01637	0.09820	0.00797	0.16096	0.00917	0.03428
Between seas (G_{SEA})	0.01202	0.00917	0.00104	0.00195	0.00299	0.08946	0.00440	0.16002	0.00917	0.02636
Between ports within seas (G_{PS})	0.00228	0	0.00251	0.00111	0.00158	0.00005	0.00056	0	0	0.00285
Between samples within ports (G_{SP})	0.00274	0	0.00376	0.00501	0.01181	0.00869	0.00301	0.00094	0	0.00507

the 5% of cases which would be expected by chance, and this result is in accordance with that obtained for north-western Mediterranean anchovies by Garcia *et al.* (1994). The indication is that breeding within those groups of fish sampled was panmictic. Samples were taken by commercial vessels using technology typical of the fishing grounds, i.e. light seine (*lampara*) and mid-water pair trawling (*volante*). The former vessels probably target single anchovy shoals, and from this we may infer that anchovy shoals are representative of larger panmictic units, or that any degree of inbreeding which occurs within these shoals is insufficient to influence Hardy-Weinberg equilibrium at allozyme loci.

Geographic heterogeneity

Within the Mediterranean there were significant differences in allelic distribution between samples. The loci *GAPDH-1** and *GPI** showed clear discontinuities across the sampling area, particularly between the Adriatic Sea and surrounding waters. *GAPDH-1** was fixed for the common allele within the Adriatic, while the frequency of the *GAPDH-1*50* allele increased from the Ionian Sea to the Tyrrhenian Sea, Sicilian Channel and Aegean Sea. *GPI** was invariable or only weakly polymorphic in Adriatic samples (*GPI*100*, 0.97–1.00), while the frequencies of variant *GPI** alleles (**130* and **50*) were far higher in the Sicilian and Aegean samples than in other locations.

*IDHP-2*100* frequencies were low in both Northern Adriatic samples (0.52 and 0.54; Trieste), but did not fall below 0.73 in any other sample except the Aegean Sea (0.62). The northern basin of the Adriatic differs considerably from the central and southern regions, being shallower and far more productive due to the input of the River Po (Bombace, 1992). Levi *et al.* (1994) reported growth differences between anchovies from the northern and central basins, pointing out that circulatory patterns in the two areas are independent and may thus form an effective stock boundary. This oceanographic discontinuity may play a role in maintaining a degree of genetic differentiation between anchovies in the two basins, and the extended spawning period and extensive spawning areas of anchovies in the region provide great scope for the persistence of temporally or spatially isolated populations. In order to further investigate these possibilities, we are currently analysing a more extensive set of Adriatic samples.

The first Ionian Sea sample showed a *GPI*100* frequency of 0.90, intermediate between those of the Adriatic (mean=0.99) and Sicilian (mean=0.80) samples, while the second sample taken two months later was invariable at this locus, a significant difference. These samples also differed at the *IDHP-2** locus. A survey of anchovy distribution in the Southern Adriatic (Casavola *et al.*, 1988) found no eggs as far south as Otranto, just outside the Gulf of Taranto where our samples were taken, and concluded that oceanographic conditions in this southern area were unsuitable for anchovy spawning. We therefore suggest that anchovies caught in the Gulf of Taranto may originate from other parts of the Ionian Sea, waters around Sicily or even the Adriatic Sea, the source population varying seasonally. Replicate samples from Adriatic ports could not be distinguished, although analysis of a more extensive dataset has revealed genetic variation between catches taken by the same sampling vessel in different months (data not shown). We feel that this is a consequence of both the mobility of the species and also of spatial variation in the fishing grounds utilized by single vessels.

Genetic distance

Nei's (1972) genetic distance (D) was very low (<0.01) for any two-way comparison, so our dendrogram was constructed using Cavalli-Sforza and Edwards' (1967) chord distance. The assumptions underlying these measures mainly differ in that Nei's model assumes an equivalent neutral mutation rate for all loci and that the effective population size remains constant, while Cavalli-Sforza and Edwards' model assumes that all gene frequency changes are due to drift alone and also accounts for changing population size (Felsenstein, 1993). Both models, however, assume that inter-population differences have arisen by genetic drift. Dobrovolov (1992) derived a Nei's (1972) distance of 0.0066 between Adriatic and Aegean *E. encrasicolus*, similar to our value of 0.0063 (mean for all six Adriatic samples), but recorded a maximum value of 0.0620 between fish from the Canary Islands and Cape Blanc, north-east Africa, arguing that these two samples represented sub-species. Such a magnitude of divergence is on a par with those between races of herring (*C. pallasi* 0.039) or cod (*Gadus macrocephalus* Til. 0.023) (Grant, 1987), but well within Thorpe's (1983) range for conspecific populations.

In our dendrogram, the Adriatic samples clustered together with the second Ionian sample, and possible reasons for their similarity have been discussed earlier. Other samples outside the Adriatic were more distinct, although, as the dendrogram construction depends on a relatively small proportion of loci (p=0.95), any comments on fine-scale relationships are inappropriate. However, the dendrogram effectively summarizes the integrity of Adriatic samples and illustrates the discreteness of this sea, connected to the main body of the Mediterranean by the relatively narrow Strait of Otranto, and characterized by its unique hydrographic features and fauna (Bombace, 1992). Estimates of renewal time for the Adriatic range from 3–10 years, with variations between the northern, central and southern basins (Umani *et al.*, 1992). Circulatory systems in

the northern and central areas are largely independent of those in the southern basin (although this varies seasonally).

Partitioning gene diversity

Total gene diversity H_T (Nei, 1973) was 0.092, greater than the mean value of 0.063 quoted for marine species by Gyllensten (1985) but not notably so, given the large error associated with this calculation (S.D.=0.121). G_{ST} averaged over all loci was 0.034, again a fairly typical value for marine teleosts. Partitioning G_{ST} indicated that diversity between seas within the Mediterranean area (G_{SEA}/G_{ST}) accounted for an average of over 76% of between sample variation, although figures for some loci were far higher (e.g. *GPI**, G_{ST}=0.098, G_{SEA}/G_{ST}=0.91; *GAPDH-1**, G_{ST}=0.161, G_{SEA}/G_{ST}=0.99), emphasizing the macro-geographic structuring of the data. Our study ranges over a considerable geographic distance, but spatial separation alone is usually too inefficient an isolating mechanism to result in significant divergence between populations of highly mobile species such as the anchovy. Indeed, Garcia *et al.* (1994) detected no significant differences at the allozyme level between anchovies from an area covering over 400 miles of the north-western Mediterranean. An underlying difference between their results and those of the current study is the relative homogeneity of the sea to the west of Corsica, there being no obvious barriers to panmixia in comparison with the hydrographic and physical structuring of waters in our study area.

Implications for stock structure

Genetic analyses are generally over-sensitive to gene flow. Relatively low levels of exchange between stocks, negligible from a management perspective, may be sufficient to ensure genetic homogeneity. Thus, Ward and Grewe (1994) make the important point that, when using allozyme electrophoresis in stock discrimination, both low and high levels of gene flow may lead to the conclusion that a single panmictic population is present. G_{ST} values, if significant (Ferguson, 1994), may be used to estimate N_eM, the effective number of migrants per generation between stocks or populations using island (Wright, 1978) or stepping-stone (Kimura and Weiss, 1964) models of population structure. Substituting G_{SEA} (the coefficient of gene differentiation between seas) for F_{ST} in $F_{ST}=1/(4N_eM+1)$ (Wright, 1943), we calculate that 8.6 migrants per generation would account for the level of differentiation observed between Mediterranean seas. Although the error associated with such an estimate is obviously substantial, it illustrates clearly the independence of anchovy stocks in the northern Mediterranean. Given such a restricted level of migration, one would predict that regeneration of depleted stocks would be almost totally autochthonous. As a result of such integrity, recovery from collapse would be slower, and the species would be more vulnerable to replacement by a competitor with a similar ecological niche when biomass levels were low. Such a scenario may have contributed to the increase in sardine (*Sardina pilchardus* Wal.) numbers in the Adriatic Sea subsequent to the environmentally-driven collapse of the anchovy fishery in the mid-1980s.

Although anchovies are commonly considered highly mobile, their localized spawning behaviour or migratory patterns may result in restricted gene flow, especially among waters of contrasting hydrography. It was not possible to delimit stock boundaries from this study alone, although the existence of genetic differences, if temporally persistent, would provide the potential for effective stock discrimination (Carvalho and Hauser, 1994). In view of this, and given the considerable commercial value of Mediterranean anchovy fisheries, continued monitoring of the temporal stability of the spatial differentiation noted here would seem an obvious research priority. Indeed, electrophoretic analysis of samples taken from the Adriatic fleet over a two year period has already revealed that allele frequencies at those loci which differentiate between fish from this sea and those in surrounding waters (*GAPDH-1**, *GPI**) are relatively invariable (Carvalho *et al.*, 1994).

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Appendix 1. Electrophoresis buffer recipes.

Buffer	Electrode buffer (E) (l^{-1})	Gel buffer (l^{-1})
Tris maleic pH 7.4 or 8.9	12.1 g Tris 11.6 g maleic acid 3.7 g EDTA 4.05 g $MgCl_2$ Adjust pH with NaOH	Dilute E 1:9
Citrate morpholine pH 7.4	8.4 g citric acid Adjust pH with *N*-(3-aminopropyl)-morpholine	Dilute E 1:9
Tris citrate pH 8.0	30.3 g Tris 11.98 g citric acid Adjust pH with HCl	Dilute E 1:25
Ridgeway's pH 8.6	4.2 g lithium hydroxide 14.9 g boric acid	3.6 g Tris 1.05 g citric acid
Poulik's pH 8.2–8.7[1]	18.6 g boric acid 2.4 g NaOH	9.2 g Tris 1.05 g citric acid

[1]P. W. Shaw, University of Wales, Swansea, pers. comm.