Stock discrimination among European anchovies, *Engraulis encrasicolus*, by means of PCR-amplified mitochondrial DNA analysis

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Abstract.—A study of mitochondrial DNA (mtDNA) variation in European anchovies, *Engraulis encrasicolus* L., in the northwestern Mediterranean area was carried out with samples from Trieste, Ancona, and Vieste in the Adriatic Sea, from the Ionian, Tyrrhenian, and Aegean seas, and the from the Sicilian Channel. Restriction fragment length polymorphisms (RFLP's) in genes of the NADH dehydrogenase complex (ND genes) were investigated by using the polymerase chain reaction (PCR). Restriction of a 2.5 Kb PCR product coding for ND5 and ND6 revealed 53 composite haplotypes in 140 fish, 39 of which were unique. Mean haplotype diversity was 0.88; hence levels of variation were notably high. Pairwise values of nucleotide divergence ranged between 0.00001 and 0.01375, and gene diversity analysis indicated that an average of 7.6% of variation was partitioned between samples (G_{ST}). There was significant geographic heterogeneity, and Monte Carlo χ^2 simulations showed that Aegean Sea fish differed significantly from most other samples. Significant differences in the distribution of restriction patterns for one enzyme (*Sau* 96I) indicated that a degree of heterogeneity may also exist between anchovy populations in the Adriatic Sea and adjacent waters, although there was no intersample variation within the Adriatic Sea. Overall, the data were in accordance with the results of previous work using allozyme electrophoresis and meristics.

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The European anchovy, *Engraulis encrasicolus* L., a shoaling clupeoid fish, is distributed along the eastern Atlantic coast from Scandinavia to West Africa, and is also found in the Mediterranean, Black, and Azov seas (Whitehead et al., 1988). A commercially important species, the anchovy represented around 25% of the Italian pelagic catch in the early 1980's (Bombace, 1992). However, the Adriatic Sea anchovy stock, an important contributor to the Italian harvest, suffered a collapse from environmental causes in 1986–87, and catches have not returned to former levels. As a consequence, market values of anchovies have increased tenfold (Cingolani et al.[1]).

The paucity of data on the stock structure of *E. encrasicolus* in the Mediterranean area belies the economic significance of the species. The majority of studies have been executed by using phenotypic characters, and Levi et al. (1994) have described two putative stocks in the Adriatic Sea that have different growth rates, as measured by otolith reading. Recently, Garcia et al.[2] reported no genetic structuring

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1 Cingolani, N., G. Kirkwood, G. Giannetti, E. Arneri, and D. Levi. 1994. Note on the stock assessment of *Engraulis encrasicolus* (L.) and *Sardina pilchardus* (Walb.) of the Northern and Central Adriatic Sea. Unpubl. manuscr. submitted to FAO-CGPM: third technical consultation on stock assessment in the Central Mediterranean; Tunis, 8–12 November 1994. Available from FAO, Viale delle Terme di Caracalla, 00100 Rome, Italy.

2 Garcia, A., I. Palomera, B. Liorzou, O. Giovanardi, and C. Pla. 1994. Northwestern Mediterranean anchovy: distribution, biology, fisheries and biomass estimation by different methods. Final Project Report to the European Community MA.3.730. Available from European Commission, Directorate-General XIV Fisheries, internal resources, Conservation Policy and Environmental Questions, Rue de la Loi 200, B-1049 Brussels, Belgium.

of anchovies after an allozyme study that covered a large area of the western Mediterranean. In contrast, Spanakis et al. (1989) were able to distinguish stocks from the Ionian and Aegean seas by morphometrics and allozyme electrophoresis, noting that a degree of migration between these areas was probable.

The application of DNA technology to stock discrimination in *E. encrasicolus* is an obvious step, given the limited success of allozyme analysis, and mitochondrial DNA (mtDNA) has several distinctive properties which commend it to such investigations. Its inheritance is essentially clonal, reducing the effective population size to 1/4 of that for nuclear genes; mtDNA variation is hence more susceptible to the effects of stochastic processes (genetic drift and partial extinctions), and interpopulation variation accumulates more rapidly. This process is perhaps accelerated by the higher mutation rates often reported for mtDNA (Brown et al., 1979). Several studies have demonstrated stock differentiation in teleosts using mtDNA where none was detected at the allozyme level (see Ward and Grewe, 1994), although Ward and Grewe (1994) pointed out that the reverse is also sometimes true. It should be noted, however, that, since mtDNA acts as a single locus, the ability to screen a number of independent loci is an advantage of allozyme and nuclear DNA (nDNA) methods.

Despite their economic importance, relatively few studies of mtDNA variation in clupeoid fish have been published. Tringali and Wilson (1993) reported no significant spatial variation in the distribution of *Sardinella aurita* Val. mtDNA haplotypes in the eastern Gulf of Mexico but found that these fish were distinct from a sample taken in waters off southern Brazil. Kornfield and Bogdanowicz (1987) described geographic heterogeneity in Atlantic herring, *Clupea harengus*, although this variation has subsequently been shown to be nonsignificant (Roff and Bentzen, 1989). Other clupeoid species studied include American shad, *Alosa sapidissima* (Bentzen et al., 1988; Nolan et al., 1991; Chapman et al., 1994) and menhaden, *Brevoortia tyrannus* and *Brevoortia patronus* (Bowen and Avise, 1990), three species whose life history strategies include estuarine or freshwater components. The only published study on *E. encrasicolus* mtDNA variation concentrated on the occurrence of heteroplasmy (multiple forms of mtDNA within an individual), attributed to "paternal leakage" (Magoulas and Zouros, 1993).

Until recently, analysis of mtDNA involved laborious extraction protocols (e.g. Lansman et al., 1981) or hybridization of mtDNA probes with Southern-blotted digests of total DNA, but the revolution in molecular biology due to the discovery of polymerase chain reaction (PCR) amplification of DNA (Saiki et al., 1988) has largely removed these obstacles. Researchers may now apply the technique to concentrate efforts on mtDNA regions of particular interest, whether for species identification or for the detection of intraspecific variation and stock markers (Chow et al., 1993; Cronin et al., 1993; Chapman et al., 1994).

The genes of the NADH dehydrogenase complex (ND genes) have been the subject of a number of recent investigations, usually exhibiting sufficient variation to provide useful genetic markers (Cronin et al, 1993; Hall, 1993; Park et al., 1993). In this study we have investigated the utility of restriction fragment length polymorphism (RFLP) analysis of PCR-amplified mtDNA ND genes in the study of population structure in anchovies from the Adriatic Sea and surrounding waters. Our sampling strategy has enabled the comparison of results from this rapid, state-of-the-art approach with those previously obtained by allozyme analysis.

Materials and methods

The anchovy samples used for analysis were from the ports of Trieste, Ancona, and Vieste in the Adriatic Sea, from the Sicilian Channel, and from the Ionian, Tyrrhenian, and Aegean seas (Fig. 1). Each sample comprised 20 individuals. Fish were captured by commercial vessels with technology typical of the fishing grounds, i.e. light seine (*lampara*) and pair trawl (*volante*), and immediately placed on solid CO_2 (dry ice). Fish were later stored at –80°C until laboratory analysis.

DNA extraction

Further details on the preparation of solutions may be found in Sambrook et al. (1989). A piece of muscle tissue approximately 5×3×3 mm was added to 300 µL extraction solution (0.1 M Tris, 0.01 M EDTA, 0.1 M NaCl, 2% SDS, proteinase K 0.8 $mgmL^{-1}$, pH 8.0), mixed, and incubated at 55°C for 1 hour. After digestion and phenol extraction, 2.5 volumes of ice-cold 99% ethanol were added, the tube was inverted several times, and spun for 5 minutes in a microcentrifuge to pellet the precipitated DNA. The pellet was washed with 70% ethanol, air-dried for 30 minutes, and dissolved in 100 µL TE buffer (0.01 M Tris, 0.02 M EDTA, pH 7.5).

PCR amplification of mtDNA

The main set of PCR primers employed were "universal" vertebrate sequences which amplified a 2.5

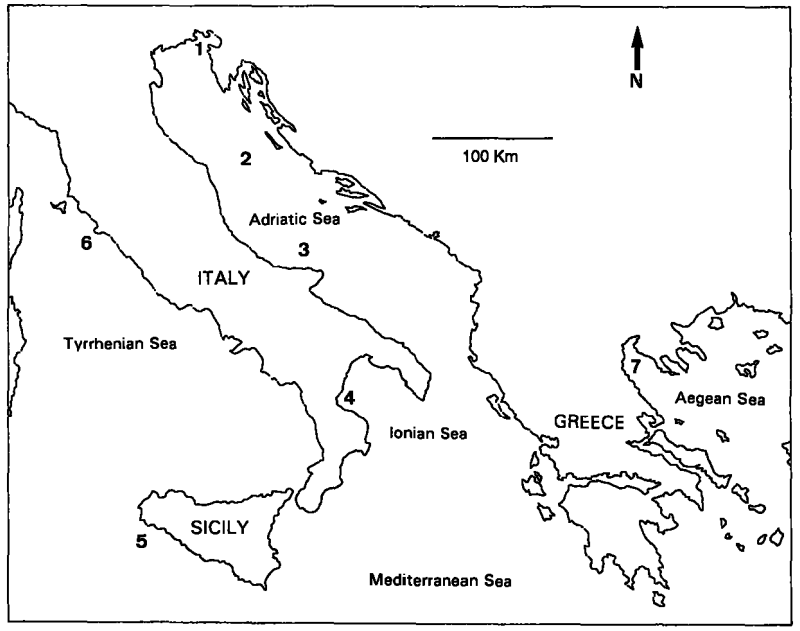

Figure 1

Map of the northern Mediterranean showing sample locations for European anchovy, *Engraulis encrasicolus*. Samples referred to in the text were Trieste (1), Ancona (2), Vieste, (3), Ionian Sea (4), Sicilian Channel (5), Tyrrhenian Sea (6), and Aegean Sea (7).

Kb region containing the ND5 and ND6 genes of the NADH dehydrogenase complex (Cronin et al., 1993).

ND 5/6
5'- AAT AGT TTA TCC AGT TGG TCT TAG -3' 24 mer
5'- TTA CAA CGA TGG TTT TTC ATA GTC A -3' 25 mer

Other mtDNA primers assayed amplified a 2.3 Kb region coding for the ND3/ND4 genes (Cronin et al., 1993) and a 2.0 Kb fragment coding for ND1 and 16s RNA (Hall, 1992[3]).

ND 3/4
5'- TAA (C/T)TA GTA CAG (C/T)TG ACT TCC AA -3' 23 mer
5'- TTT TGG TTC CTA AGA CCA A(C/T)G GAT -3' 24 mer

ND1/16s
5'- ACC CCG CCT GTT TAC CAA AAA CAT -3' 24 mer
5'- GGT ATG AGC CCG ATA GCT TA -3' 20 mer

The amount of template DNA for the PCR reaction was usually 1 μL of the 100 μL volume extraction described above (approximately 50 ng), but occasionally a 1/10 dilution was required for efficient amplification. The reaction cocktail (per 50 μL reaction) contained 5 μL 10× PCR buffer (0.5 M KCl, 0.1 M Tris, 2.5 mM $MgCl_2$, pH 8.3), 5μL dNTP mix (at 2 mM with respect to each dNTP), 1 μL of each primer (approximately 25 pmol), 1 u Boehringer *Taq* polymerase, 1 μL template DNA and 37 μL sterile filtered dH_20. This was overlaid with two drops of sterile mineral oil. Amplification cycle conditions for the ND5/6 primers, with the use of a Hybaid Omni-Gene thermal cycler, were 1) 95°C, 5 min; 1 cycle; 2) 49°C, 1 min 30 s; 72°C, 1 min 30 s; 94°C, 30 s; 25 cycles; 3) 49°C, 1 min 30 s; 72°C, 10 min; 1 cycle. Conditions for amplifying ND1/16s and ND3/4 were identical, except that, for ND1/16s, annealing took place at 51°C.

DNA restriction and data collection

From 3 to 5 μL of PCR product were restricted with a range of endonucleases recognizing four, five, and six nucleotide sequences: *Aat* II, *Alu* I, *Ava* I, *Ava* II, *Cfo* I, *Eco* RI, *Hae* III (*Pal* I), *Hind* III, *Hinf* I, *Msp* I, *Nci* I, *Pvu* I, *Rsa* I, *Sau* 3AI, *Sau* 96I, and *Taq* I. Restriction products were resolved on agarose and polyacrylamide gels by using a TBE buffer system (Sambrook et al., 1989) and visualized by ethidium bromide and silver nitrate staining, respectively. Restriction fragment data were recorded from all gels, and fragment sizes estimated from their mobilities relative to lambda-phage DNA-marker frag-

[3] Hall, H. 1992. Zoological Society of London, Regent's Park, London, U.K. Personal commun.

ments. For each enzyme, the various restriction patterns were named alphabetically ("A" being the most common), and the resultant composite haplotypes were assigned numerically. Within-sample variation was estimated as nucleotide diversity (Nei and Tajima, 1981; Nei, 1987), as the average number of nucleotide substitutions per site for the sequences sampled, and as haplotype diversity, which is related to allozyme heterozygosity. Nucleotide divergence (Nei, 1987) was used to measure between-sample variation. The significance of geographic heterogeneity in haplotype distribution was tested by using a Monte Carlo χ^2 approach (Roff and Bentzen, 1989), and this method was also used to test the distribution of banding patterns produced by single restriction enzymes. Partitioning of mtDNA variation was accomplished by gene diversity analysis (Nei, 1973; Chakraborty, 1980). Between-sample variation (G_{ST}) was subdivided into variation between seas (G_{SEA}) and between sampling ports within seas (G_{PS}). Data were analysed by using REAP v4.1 (McElroy et al., 1992), PHYLIP v3.5c (Felsenstein, 1993), and spreadsheet macros were written for Lotus 1-2-3.

Results

DNA amplification

Of the three primer pairs assayed, only those for the ND5/6 and ND1/16s regions produced amplification products consistently. No products were obtained from anchovy DNA templates with the ND3/4 primer set. We estimated the sizes of the unrestricted ND5/6 and ND1/16s PCR products to be 2.5 Kb and 2.0 Kb, respectively.

Levels of variability

In a preliminary study of 15 fish, 10 ND5/6 composite haplotypes were revealed by using 12 restriction enzymes (***Alu* I**, *Ava* II, ***Cfo* I**, ***Eco* RI**, ***Hinf* I**, *Msp* I, *Nci* I, *Pal* I, *Rsa* I, ***Sau* 96I** and ***Taq* I**). Only one enzyme, *Sau* 3AI, was monomorphic for all samples; all other enzymes revealed variation. The same number of haplotypes revealed by these eleven enzymes could also be differentiated by using a subset of six (those in bold type); hence these six were chosen to analyze the entire sample set.

Initial studies indicated that levels of diversity in ND1/16s were of a similar order to those in ND5/6. Sixteen endonucleases were used (*Aat* II, ***Alu* I**, *Ava* I, ***Ava* II**, ***Cfo* I**, *Eco* RI, ***Hae* III**, *Hind* III, *Hinf* I, ***Msp* I**, ***Nci* I**, *Pvu* I, ***Rsa* I**, *Sau* 3AI, ***Sau* 96I**, *Taq* I), five of which did not cut the fragment (*Aat* II, *Ava* I, *Eco* RI, *Hind* III, and *Pvu* I), three of which produced monomorphic profiles (*Hinf* I, *Sau* 3AI, and *Taq* I), and another eight of which revealed polymorphism (bold type). Ten composite haplotypes were detected in 15 individuals. Because of logistic considerations, further work on the ND1/16s region was sacrificed in favor of increasing sample numbers in the study of ND5/6 variation; hence all subsequent results refer solely to the latter region.

A total of 77 ND5/6 restriction fragments were scored for the entire data set of 140 fish, revealing 53 composite haplotypes (Table 1). Of these, 14 haplotypes occurred at least twice, and the remaining 39 were encountered in a single fish only. Only haplotype 3 was found at all sample locations. Mean within-sample haplotype diversity was 0.8816 (±0.0004), and mean nucleotide diversity was 0.0164 ($\pm 3\times10^{-6}$) (Table 2). According to both measures, the lowest level of variation was found in the Aegean Sea sample, where 45% of the fish were of genotype 3.

Inter-sample variation

Nucleotide divergence between samples varied between 0.0005 and 0.0137, the mean figure being 0.0031 (Table 3). The greatest values of nucleotide divergence were in comparisons involving the Aegean Sea sample.

Monte Carlo χ^2 analysis of geographic heterogeneity was, in each case, carried out with 1,000 randomizations of the data set. When all samples and all 53 haplotypes were included, there was significant geographic heterogeneity (Table 4(i); P=0.011), and this was also true of the distribution of restriction morphs for all enzymes except *Eco* RI (data not shown). Removing the Aegean Sea group resulted in a probability of geographic homogeneity of haplotypes of 0.337 (Table 4(ii)). Indeed, out of 21 pairwise comparisons between all seven samples (Table 5), only five were significant at $P<0.05$, and these all involved the Aegean sample (only the Aegean–Sicilian-Channel comparison was nonsignificant; P=0.23). Sequential Bonferroni testing (Rice, 1989) rejected all but the two most significant of these results, however (Table 5).

A comparison of the distribution of 28 composite haplotypes between the three Adriatic samples was nonsignificant (P=0.689; Table 4(iii)), hence the Adriatic samples were pooled (n=60). Although there was no significant heterogeneity in haplotype distribution between the pooled Adriatic and outlying samples (omitting the Aegean Sea) (P=0.488; Table 4(iv)), there was significant heterogeneity in the distribution of forms revealed by one out of the six enzymes (Table 4(v); *Sau* 96I, P=0.007). There was thus an indication of genetic heterogeneity between Adriatic fish and those from adjacent waters.

Table 1

European anchovy, *Engraulis encrasicolus*, mtDNA composite haplotypes, and their distribution across samples. Enzymes used to constuct composite haplotypes were (left to right): *Alu* I, *Cfo* I, *Eco* RI, *Hinf* I, *Sau* 96I and *Taq* I. Sample locations were Trieste (Tr), Ancona (An), Vieste (Vi), Ionian Sea (Io), Sicily (Si), Tyrrhenian Sea (Ty) and Aegean Sea (Aeg).

		Number of each haplotype per sample						
	Haplotype	Tr	An	Vi	Io	Si	Ty	Aeg
1	AAAAAB	4	2	1	2	1	5	—
2	AAAABA	1	3	2	2	1	2	—
3	BBABCC	3	2	2	6	7	5	9
4	ACAABA	3	1	1	—	—	—	—
5	AAAADB	1	1	3	1	2	3	—
6	AAAABC	—	1	1	—	—	—	—
7	DABAAB	2	1	—	—	—	—	—
8	EABAAB	—	3	1	1	—	—	—
9	DABFAB	1	—	—	1	—	—	—
10	AAACAB	—	1	1	1	—	—	—
11	CDBDAB	—	1	—	2	1	—	—
12	IBABCC	—	—	—	—	—	—	2
13	BGABEC	—	—	—	—	1	—	1
14	BBABKC	—	—	—	—	—	2	—
15	DABAAA	1	—	—	—	—	—	—
16	FABAAB	1	—	—	—	—	—	—
17	GCAABA	1	—	—	—	—	—	—
18	AFAABA	1	—	—	—	—	—	—
19	BBAGCA	1	—	—	—	—	—	—
20	BCAABA	—	1	—	—	—	—	—
21	AHABCC	—	1	—	—	—	—	—
22	DABAAC	—	1	—	—	—	—	—
23	ACCABA	—	1	—	—	—	—	—
24	HBABCC	—	—	1	—	—	—	—
25	CDBAAB	—	—	1	—	—	—	—
26	IBABCC	—	—	1	—	—	—	—
27	AEACAB	—	—	1	—	—	—	—
28	AAACAG	—	—	1	—	—	—	—
29	EAAABA	—	—	1	—	—	—	—
30	AAAHAB	—	—	1	—	—	—	—
31	BCABCC	—	—	1	—	—	—	—
32	BBABCF	—	—	—	1	—	—	—
33	BBABEC	—	—	—	1	—	—	—
34	AAAAIC	—	—	—	1	—	—	—
35	BBABJC	—	—	—	1	—	—	—
36	AEBIAB	—	—	—	—	1	—	—
37	BAAADB	—	—	—	—	1	—	—
38	AAAAHD	—	—	—	—	1	—	—
39	AAAAGB	—	—	—	—	1	—	—
40	AAAAFA	—	—	—	—	1	—	—
41	BBAECE	—	—	—	—	1	—	—
42	DCBAAB	—	—	—	—	1	—	—
43	BBABCE	—	—	—	—	—	1	—
44	IBABCC	—	—	—	—	—	1	—
45	BBABIE	—	—	—	—	—	1	—
46	BBAICC	—	—	—	—	—	—	1
47	BHABKC	—	—	—	—	—	—	1
48	BBABCA	—	—	—	—	—	—	1
49	IIABCC	—	—	—	—	—	—	1
50	IABAAB	—	—	—	—	—	—	1
51	BBBBCC	—	—	—	—	—	—	1
52	BJABCC	—	—	—	—	—	—	1
53	BHABCC	—	—	—	—	—	—	1

Gene diversity analysis of all seven samples (Table 6) indicated that over 92.4% of the mtDNA variation detected was within samples, hence 7.6% was partitioned between samples (G_{ST}). Jackknifed estimates of G_{ST} were in the 5.8–8.3% range, and the mean estimate was significantly different from zero (t_s=22.51, df=6, P<0.001). G_{ST} values for pairwise comparisons of samples were in the 2.6–19.4% range, the highest values from comparisons involving the Aegean Sea group (Table 7). G_{ST} was subdivided into variation between seas (G_{SEA}) and between sampling ports within seas (for G_{PS}, in the Adriatic only). G_{SEA} accounted for 77% of inter-sample variation, leaving 23% partitioned between Adriatic ports (Table 6). The indication was that the degree of genetic differentiation was greater on a larger geographic scale.

Discussion

Restriction analysis of PCR-amplified mtDNA is a relatively simple technique which enables the rapid screening of large numbers of fish for DNA-level polymorphism. The wide range of "universal" and teleost-specific primer sequences in the literature (e.g. Avise, 1994) means that one or more fragments that amplify successfully should be obtainable for most species. Additionally, the production of μg quantities of DNA by PCR and the silver-staining technique remove the necessity for isotopic labelling of restriction fragments, an improvement both in terms of cost and safety.

Our results show that variability in anchovy ND5/6 genes is notably high: 53 haplotypes were detected in only 140 fish, with a mean nucleotide diversity of 0.0164. Hauser et al. (in press) have reported similar levels of variation for the ND5/6 region in kapenta, *Limnothrissa miodon,* a freshwater clupeoid. Using the same primers as those in this study and a set of six enzymes, they reported 85 haplotypes in 362 fish, with nucleotide diversities of 0.0137 and 0.0098 for Lake Tanganyika and Lake Kivu fish, respectively, and commented that the order Clupeiformes may show generally high levels of mtDNA variation. These figures contrast with a restriction study that showed the ND5/6 region to be monomorphic in Atlantic salmon, *Salmo salar* (O'Connell, 1993), while work on brown trout, *Salmo trutta* (Hall, 1993), has demonstrated intermediate levels of variation (19 haplotypes in 219 fish with the use of seven enzymes). Recent work on the ND5/6 genes of Trinidadian guppies, *Poecilia reticulata,* has also shown low levels of intrapopulation diversity, attributed to the effects of local extinctions and founder events (Shaw[4]). We believe that the relatively high level of variation described here may be representative of the entire anchovy mtDNA genome because our pilot study of the ND1/16s region showed that variability was of a similar order to ND5/6. Chapman et al. (1994), in a recent study of the genus *Alosa*, noted that the ND1/16s region evolved more quickly than cy-

Table 2

Levels of mtDNA variation within samples of European anchovy, *Engraulis encrasicolus,* measured as haplotype and nucleotide diversity.

Sample	Haplotype diversity	Nucleotide diversity
Trieste	0.9077 ±0.02177	0.017130
Ancona	0.9333 ±0.01703	0.017974
Vieste	0.9487 ±0.01493	0.019318
Ionian	0.8821 ±0.03557	0.020577
Sicily	0.8615 ±0.04568	0.017546
Tyrrhenian	0.8462 ±0.02839	0.015874
Aegean	0.7846 ±0.06273	0.006370
Mean	0.8806 ±0.00045	0.016399

[4] Shaw, P. W. 1994. School of Biological Sciences, Univ. Wales Swansea, Singleton Park, Swansea, U.K. Personal commun.

Table 3

Nucleotide divergence (Nei, 1987 [Eq. 10.21]) between samples of European anchovy, *Engraulis encrasicolus.*

	Nucleotide divergence					
Sample	Trieste	Ancona	Vieste	Ionian	Sicily	Tyrrhenian
Ancona	0.00040					
Vieste	0.00053	0.00032				
Ionian	0.00001	0.00011	0.00042			
Sicily	0.00302	0.00333	0.00160	0.00060		
Tyrrhenian	0.00216	0.00247	0.00105	0.00031	0.00046	
Aegean	0.01281	0.01375	0.01032	0.00799	0.00286	0.00423

Table 4

Monte Carlo χ^2 simulation analyses of the geographical distribution of mtDNA variation in European anchovy, *Engraulis encrasicolus*. For all samples n=20. For all analyses, 1,000 iterations were performed. * = those comparisons that were still significant at P<0.05 after sequential Bonferroni testing.

i) Comparing all seven samples; 53 composite haplotypes
χ^2 from original matrix = 323.3, exceeded by 11/1,000 simulations; P=0.011*;

ii) Comparing all samples except Aegean Sea; 44 composite haplotypes
χ^2 from original matrix = 224.5, exceeded by 337/1,000 simulations; P=0.337;

iii) Comparing the three Adriatic Sea samples only; 28 composite haplotypes
χ^2 from original matrix = 51.99, exceeded by 689/1,000 simulations; P=0.689;

iv) Comparing all samples except the Aegean Sea after pooling Adriatic Sea samples (i.e. four sample groups); 44 composite haplotypes
χ^2 from original matrix = 130.41, exceeded by 488/1,000 simulations; P=0.488;

v) Comparisons of the distribution of restriction patterns for individual endonucleases between a pooled Adriatic sample (n=60) and all other groups except the Aegean Sea.

Enzyme	No. patterns	Original χ^2	Exceeded by n simulations	P
Alu I	9	22.5	588/1,000	P=0.588
Cfo I	8	23.5	321/1,000	P=0.321
Eco RI	3	10.1	100/1,000	P=0.100
Hinf I	9	31.7	111/1,000	P=0.111
Sau 96I	11	49.4	7/1,000	P=0.007*
Taq I	8	31.3	48/1,000	P=0.048

Table 5

Probability values for pairwise Monte Carlo χ^2 comparisons of ND5/6 composite haplotype distribution in samples of European anchovy, *Engraulis encrasicolus*. All comparisons employed 1,000 iterations. * = those comparisons that were still significant at P<0.05 after sequential Bonferroni testing.

Sample	Trieste	Ancona	Vieste	Ionian	Sicily	Tyrrhenian
Ancona	0.463					
Vieste	0.347	0.952				
Ionian	0.282	0.815	0.620			
Sicily	0.116	0.248	0.474	0.807		
Tyrrhenian	0.151	0.100	0.242	0.302	0.272	
Aegean	<0.001*	<0.001*	0.003	0.022	0.231	0.003

tochrome *b*, and an alternative view is that the genes of the NADH dehydrogenase system have a uniformly high rate of evolution. Magoulas (1993), however, has reported over 30 composite haplotypes in RFLP analysis of the entire *E. encrasicolus* mtDNA molecule, and, because his results were obtained by using five enzymes which recognized hexanucleotide sites only, his observations lend support to our hypothesis.

The distribution of both composite haplotypes and restriction forms for individual restriction enzymes differed between the Aegean Sea anchovy population and the other areas sampled. When the same samples were studied with allozyme electrophoresis, the greatest estimates of genetic divergence were also found in comparisons involving Aegean Sea fish; hence both techniques confirm the distinctness of this group (Carvalho et al.[5]). Magoulas (1993) found two

[5] Carvalho, G. R., D. G. Bembo, A. Carone, G. Giesbrecht, N. Cingolani, and T. J. Pitcher. 1994. Stock discrimination in relation to the assessment of Adriatic anchovy and sardine fisheries. Final Report to the European Community EC XIV-1/MED/91/001/A. Available from European Commission, Directorate-General XIV Fisheries, internal resources, Conservation Policy and Environmental Questions, Rue de la Loi 200, B-1049, Brussels, Belgium.

Table 6

Gene diversity analysis of ND5/6 haplotypes in European anchovy, *Engraulis encrasicolus*. Minima and maxima were derived by jackknife deletion of samples (i.e. seven recalculations).

Gene diversity		Mean	Minimum	Maximum
Total	H_T	0.9327	0.9247	0.9377
Within samples	H_S	0.8615	0.8475	0.8750
Between samples	D_{ST}	0.0712	0.0541	0.0782
Coefficients				
Within samples	G_S	0.9236	0.9164	0.9412
Between samples	G_{ST}	0.0763	0.0583	0.0836
Between seas	G_{SEA}	0.0584	0.0395	0.0687
Between ports within seas	G_{PS}	0.0178	0.0148	0.0241
Mean G_{SEA}/G_{ST} =76.6%				
Mean G_{PS}/G_{ST} =23.4%				

Table 7

G_{ST} values for pairwise comparisons of samples of European anchovy, *Engraulis encrasicolus*.

	Coefficient of between-sample variation G_{ST}					
Sample	Trieste	Ancona	Vieste	Ionian	Sicily	Tyrrhenian
Ancona	0.0966					
Vieste	0.0261	0.0774				
Ionian	0.1202	0.1081	0.1014			
Sicily	0.1301	0.1185	0.1111	0.1403		
Tyrrhenian	0.1363	0.1251	0.1179	0.1479	0.1576	
Aegean	0.1667	0.1553	0.1480	0.1766	0.1856	0.1945

main anchovy mtDNA phylads (groups of related haplotypes) in the Mediterranean: phylad *A* dominated the Black and Aegean seas, and phylad *B* was more numerous in western waters. More extensive sampling in the Aegean and Black seas would allow us to test whether the same groupings are supported by analysis of the ND5/6 genes only.

The effective number of female migrants between populations, N_eM, may be estimated from mtDNA data by using the term $N_eM = (1/G_{ST}-1)/2$ (Nei, 1973; Ward and Grewe, 1994). On the basis of our data from pairwise comparisons, between 2 and 20 migrant females per generation could account for observed levels of interpopulation diversity. The former figure was calculated for the comparison between Aegean Sea and Tyrrhenian Sea fish, the two most spatially isolated samples. Such data should always be interpreted with caution, however (Ferguson, 1994), because small (and nonsignificant) values of G_{ST} will still generate estimates of numbers of effective migrants that will appear low relative to estimates of stock size for pelagic species. For example, the higher figure of 20 quoted above was derived from a G_{ST} value of 0.0261, although Monte Carlo χ^2 analysis had not demonstrated significant heterogeneity between the samples.

All Monte Carlo comparisons of haplotype distribution that omitted the Aegean Sea sample were nonsignificant, although, after pooling the three Adriatic samples, a comparison of restriction pattern distribution was significant for one enzyme (*Sau* 96I). There was thus some indication of genetic differentiation between fish from the Adriatic Sea and adjacent waters, as has already been suggested by allozyme analysis (Carvalho[5]). The discreteness of the Adriatic stock has also been demonstrated with more traditional methods, and meristic studies have shown that these anchovies are highly distinct from those found in the neighboring Ionian Sea (Carvalho[5]). The large number of unique, composite haplotypes found in the ND5/6 genes undoubtedly confounded analysis, and further work on *E. encrasicolus*

ND5/6 genes will require greater sample sizes (probably around 40–50 fish). Alternatively, another region such as ND1/16s or the cytochrome oxidase (CO) genes might be a source of complementary mtDNA markers. In revealing spatial trends in accordance with those recorded in previous allozyme and mtDNA studies, our results underline the power, sensitivity, and speed of this genetic approach to stock discrimination in such a highly mobile marine teleost.

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