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Phylogeography of the Atlantic bonito (*Sarda sarda*) in the northern Mediterranean: the combined effects of historical vicariance, population expansion, secondary invasion, and isolation by distance

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Abstract

Sequence analysis of the mtDNA control region of four samples ($n = 195$) of Atlantic bonito (*Sarda sarda*) collected along the northern Mediterranean reveals two clades about 8.1% divergent distributed in an east-west cline that fits an isolation by distance (IBD) model. The vicariant origin of this genetic discontinuity is proposed, supported in addition to the cline, by evidence of distinct historical demographic factors affecting each clade. Variation in Clade I suggests a large stable population, whereas Clade II displays a star-like phylogeny indicative of a population bottleneck followed by sudden expansion. The historical demography and biogeographic scenario is as follows: (1) Allopatric isolation during the Pleistocene give rise to Clade I (Atlantic) and Clade II (Mediterranean); (2) Population collapse followed by sudden expansion gives rise to the characteristic star-like phylogeny of Clade II; (3) Secondary contact as Clade I enters from the Atlantic, and (4) An east-west cline is maintained by IBD.

Keywords: Atlantic bonito; *Sarda sarda*; Mitochondrial DNA control region; Stock structure; Isolation by distance; Phylogeography; Vicariance; Mismatch distribution; Mediterranean Sea

1. Introduction

Atlantic Bonito (*Sarda sarda* Bloch, 1793)—the only member of the genus *Sarda* distributed along the tropical and temperate coasts of the Atlantic Ocean, the Mediterranean Sea, and the Black Sea (Collette and Chao, 1975)—inhabits pelagic neritic waters about 200 m deep (Yoshida, 1980). Although little is known about its reproductive biology, based on the distribution of eggs, larvae, and mature individuals, two main spawning areas have been defined both geographically and temporally within the Mediterranean. The first area located in the western Mediterranean include the Alboran Sea, the waters south of the Balearic Islands and the waters off the Algerian coast where spawning occurs during the early summer (Rey et al., 1984; see Fig. 1 in Pujolar et al., 2001). The second area is located at the eastern extreme of the Mediterranean and includes the coastal waters of the Sea of Marmara and the Black Sea with spawning occurring from June and July (Yoshida, 1980). A tag-recapture study of Atlantic bonito in the Mediterranean detected no exchange of fish between the east and west, and thus putatively between the two known reproductive areas. Based on all this information, Rey et al. (1984) hypothesized that two isolated sub-populations exist in the Mediterranean. However, recently an additional spawning area has been identified in northern Balearic Sea (Sabatés and Recasens, 2001) suggesting the possibility that additional spawning areas exist.

The proposed population subdivision of Atlantic bonito in Mediterranean has been partially corroborated by the molecular genetic studies of Roberti et al.

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(1993) and Pujolar et al. (2001). However, these studies disagree on the placement of a putative barrier that prevents gene flow between eastern and western populations. Roberti et al. (1993) conducted an analysis of 300 bp segment of the mitochondrial DNA (mtDNA) Cytochrome *b* (Cyt*b*) gene. A small but significant difference in haplotype frequency was found between a sample from the Sea of Marmara and two Mediterranean samples, one from the Aegean Sea and the other from the Ionian Sea, which in turn were not different from each other. Roberti et al. (1993) concluded that a barrier prevents gene flow between the Sea of Marmara and Aegean Sea. Interestingly, these two regions are considered, respectively, spawning and feeding areas for the eastern population. In the protein electrophoresis study of Pujolar et al. (2001), a small but significant differentiation between the Aegean Sea and two western samples, namely the Ionian Sea and the Ligurian Sea was found. Although a sample from the Sea of Marmara was not included, Pujolar et al. (2001) argued that the barrier to gene flow between east and west is located in the region that separates the Ionian and the Aegean seas. Thus, allozyme data would appear concordant with the interpretation of Rey et al. (1984) about an eastern and western subdivision, but not concordant with the mitochondrial data of Roberti et al. (1993).

The low level of geographic genetic differentiation reported for Atlantic bonito in the Mediterranean at both nuclear and mtDNA loci is concordant with the expected weak signals of genetic differentiation for marine species due to the high gene flow potential (Waples, 1998). Conversely, substantial levels of genetic differentiation can be expected for small epibenthic marine fish. Recently, profound levels of genetic differentiation were reported for the sand goby (*Pomatoschistus minutus*) between two Mediterranean populations (Stefanni and Thorley, 2003). However, substantial genetic differentiation has been reported for the highly vagile European anchovy (*Engraulis encrasicolus*) within the Mediterranean (Magoulas et al., 1996). In both examples, historical vicariance and barriers to gene flow are invoked as the primary mechanism to explain the observed patterns of differentiation. However, vicariance and gene flow barriers are not the only mechanism capable of generating genetic differentiation, and recently isolation by distance (IBD) has been used to explain the population structure at both nuclear and mtDNA levels for several marine species that possess free living larval and/or adult stages (Nesbø et al., 2000; Pogson et al., 2001; Reeb et al., 2000; Wirth and Bernatchez, 2001). In these studies, the genetic differentiation arises from the reduction of the gene flow as geographic distance increases rather than the presence of a barrier to gene flow.

In the present study, we examined the genetic basis of the population structure of Atlantic bonito in the northern Mediterranean, from the DNA nucleotide sequence analysis of 405 bp of the mtDNA control region (CR). We show that there is a significant correlation between geographical distance and genetic distance resulting from the asymmetric distribution of two highly divergent mtDNA clades with very distinct demographic histories.

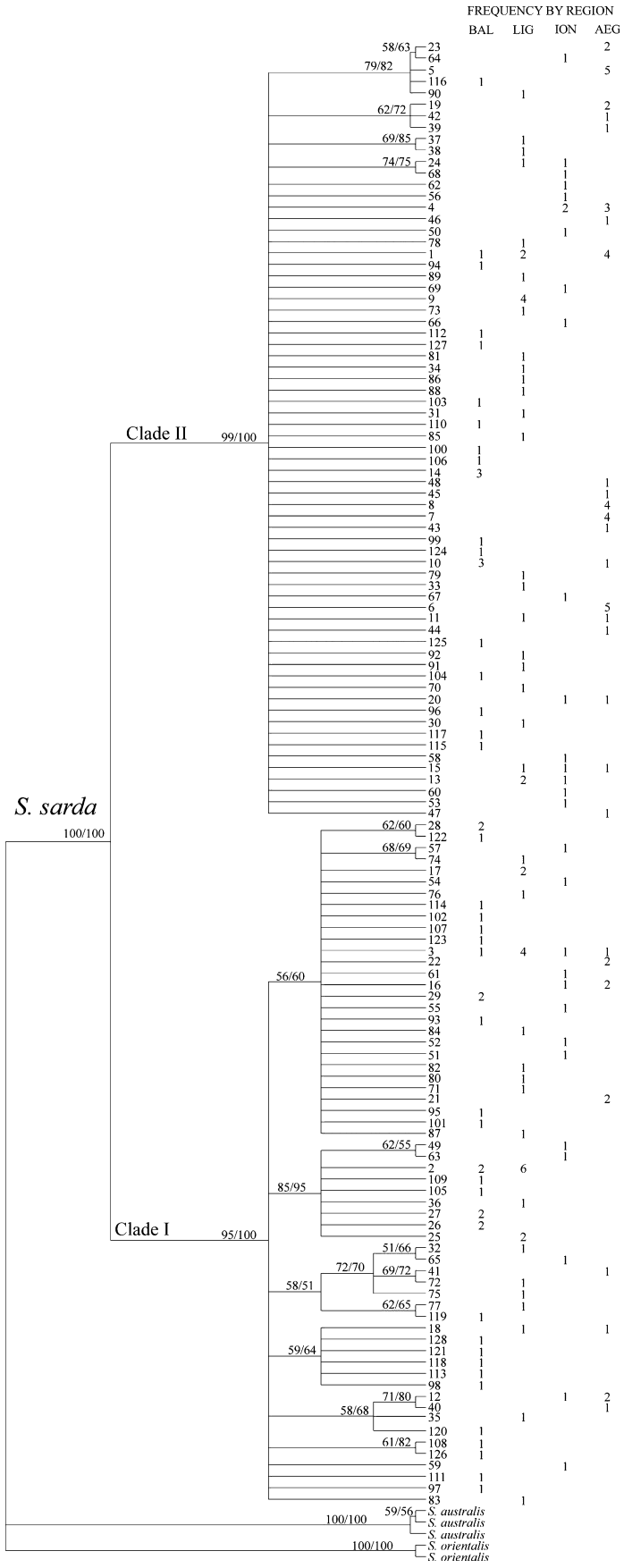
2. Materials and methods

Genetic analyses were conducted on 195 Atlantic bonito collected in the following northern Mediterranean locations: Balearic Sea ($n = 54$), Ligurian Sea ($n = 58$), Ionian Sea ($n = 36$), and Aegean Sea ($n = 53$). Sample details are included in Table 1. In addition, DNA sequences of Australian bonito *S. australis* ($n = 3$) and striped bonito *S. orientalis* ($n = 2$) were characterized and included as outgroups. Skeletal muscle or fin clips of caudal fin from each individual were collected and either preserved in 96% ethanol or frozen at −30 °C until assayed in the laboratory. Methods for DNA isolation, PCR amplification, and DNA sequencing followed Viñas et al., 2004. Sequences of all haplotypes were submitted to GenBank and assigned Accession Nos. AY526910–AY527037.

Sequence alignments were optimized by eye in BIOEDIT (Hall, 1999). The orthologous sequences of Atlantic bluefin tuna (*Thunnus thynnus*) (GenBank Accession No. X81563) and Atlantic bonito (GenBank Accession No. AF390212) were used as reference to facilitate the alignment. The multiple sequence alignment of Atlantic bonito and the outgroup revealed that one set of lineages of the former contained a pentamer insertion at the 5′-end of the control region. This insertion was weighed as one substitution in ensuing analyses. The HKY (Hasegawa et al., 1985) model with $\alpha = 0.813, i = 0.434$, transition transversion ratio (ti/tv) of 2.456 and base frequency of A: 0.343, C: 0.233, G:

Table 1
Sampling details, locations, and number of individuals (n)

Sampling locality	(n)	Location	Mean FL (cm)	Collection dates
Balearic Sea	54	41°51N–3°10E	54.1	13/11/2002
Ligurian Sea	58	43°30N–9°E	57.5	13/6/1993–6/1994
Ionian Sea	30	39°30N–17°30E	52.0	2–10/1994
Aegean Sea	53	39°N–25°30E	56.4	1–31/10/1993


FREQUENCY BY REGION
BAL LIG ION AEG
Clade II
Clade I
S. sarda
S. australis
S. orientalis

0.147, and T: 0.277 was identified as the appropriate model of substitution using a hierarchical series of likelihood ratio tests as implemented in MODEL TEST 3.06 (Posada and Crandall, 1998).

Phylogenetic analyses were performed in PAUP* 4.0b10 (Swofford, 2000). The large number of haplotypes prohibited a search for the maximum likelihood tree, so the more computationally efficient method neighbor-joining (NJ) method (Saitou and Nei, 1987) was used to construct a tree with the best-fit model of DNA evolution. Additionally, a maximum-parsimony (MP) (Fitch, 1971; Kluge and Farris, 1969) analysis conducting heuristic searches with the default options was carried out. Evaluation of statistical confidence in nodes was based in 1000 non-parametric bootstrap replicates (Felsenstein, 1985). In all phylogenetic analyses, the mtDNA CR sequences of Australian bonito and striped bonito were used as outgroups to root the tree.

The number of segregating sites (K), mean sequence divergence between groups corrected by within-group divergence (D_A) and values of haplotypic diversity (h) (Nei and Tajima, 1981) and of nucleotide diversity (π) (Nei, 1987) were computed with ARLEQUIN 2.000 (Schneider et al., 2000). Because the best-fit model is not implemented in ARLEQUIN, the Tamura-Nei (Tamura and Nei, 1993) model with the gamma parameter previously estimated was used to calculate the genetic distance between pairs of haplotypes. The proportion of variance distributed among samples was tested using the hierarchical analysis of the molecular variance procedure (AMOVA) (Excoffier et al., 1992) as implemented in ARLEQUIN. The haplotypic correlation measure (Φ_{st}) was examined considering all four samples as belonging to one population. Additionally, the haplotypic correlation measure (Φ_{st}) between each possible sample pair was estimated. Significance levels were determined by conducting a non-parametric permutation procedure 1000 times. The extent of geographic heterogeneity in allele-frequency distribution was analyzed through Monte Carlo randomizations, as described by Roff and Bentzen (1989), using the MONTE in REAP (McElroy et al., 1992). The sequential Bonferroni test (Holm, 1979; Rice, 1989) was used to correct for multiple testing.

Demographic history was examined by two approaches. First, Tajima's test of neutrality (Tajima, 1989) which compares the average number of pairwise nucleotide differences (k) between haplotypes in a sample (M) expected from the number of segregating sites (K) was used to infer the population history. A population that has been experienced population expansion may result in a rejection of the null hypothesis of neutrality. Alternatively, mismatch distribution analyses were used to evaluate possible events of population expansion and decline (Rogers, 1995; Rogers and Harpending, 1992). A population that has experienced a rapid expansion or bottleneck in the recent past shows a smooth wave-like mismatch distribution with a star-like genealogy (Rogers and Harpending, 1992; Slatkin and Hudson, 1991). All demographic analyses were computed in ARLEQUIN.

The null hypothesis of no correlation between genetic differentiation and geographic distances was tested by permuting the Mantel's non-parametric test (Mantel, 1967) 1000 times in ARLEQUIN. Alternatively, the matrix of pairwise populations Φ_{st} values generated with AMOVA was linearized (Slatkin, 1991) and regressed against the matrix of geographic distances between locations (Rousset, 1997). Geographic distances were calculated as the shortest line paths that follow the 200 m bathymetric contour based on the known spatial distribution of Atlantic bonito in the Mediterranean (see Section 1).

3. Results

Approximately 405 bp of nucleotide sequence of the mtDNA CR was determined for 195 Atlantic bonito. About 305 bp corresponded to the hypervariable first domain with the remaining 100 bp to the second or central domain of the CR. The transition/transversion ratio ($r = 2.456$) in Atlantic bonito's CR is the lowest reported to date in scombroid fishes (Alvarado Bremer et al., 1995; Alvarado Bremer et al., 1997). A total of 127 segregating sites (K), of which 80 were parsimony informative, defined 128 distinct Atlantic bonito mtDNA types. Inclusion of the outgroup sequences, *S. australis* ($n = 3$) and *S. orientalis* ($n = 2$), increased K to 158, and parsimony sites to 120. The mutation rate among sites along the CR segment characterized in Atlantic bonito was heterogeneous yielding a low alpha value ($\alpha = 0.813$). Molecular diversity indices for all sampling locations were very similar, but the lowest value consistently corresponded to the Aegean Sea sample (Table 2).

Fig. 1. Maximum-Parsimony bootstrap (MP) tree of the 128 Atlantic bonito (*Sarda sarda*) haplotypes. Branches were collapsed when the bootstrap value were less than 50%. Tree was rooted using Australian bonito (*S. australis*) and striped bonito (*S. orientalis*) as outgroup. A tree with essentially the same topology was obtained using neighbor-joining (NJ) and HKY (Hasegawa et al., 1985) model of substitution with $\alpha = 0.813, i = 0.434$, transition transversion ratio (ti/tv) of 2.456. Bootstrap support for branching is shown above branches (MP/NJ). The geographic distribution of haplotypes and their frequency is shown in the right margin. Abbreviations of localities are as follows: Balearic Sea (BAL), Ligurian Sea (LIG), Ionian Sea (ION), and Aegean Sea (AEG).

Table 2
Number of individuals (n), number of haplotypes (M), and molecular diversity indices for the four localities

Locality	n	M	Molecular diversity indices	
			h	π
Balearic Sea	54	45	0.992 (0.005)	0.071 (0.035)
Ligurian Sea	58	43	0.981 (0.009)	0.063 (0.031)
Ionian Sea	30	29	0.997 (0.009)	0.067 (0.034)
Aegean Sea	53	28	0.966 (0.010)	0.051 (0.025)
Total	195	128	0.993 (0.002)	0.063 (0.031)

h, haplotypic diversity (SD); π, nucleotide diversity (SD).

The topology of the gene trees obtained using MP and NJ analyses were concordant with the most robust branches supported by similar values of bootstrap (Fig. 1). The MP tree was 419 steps long and had a high retention index (RI = 0.895). The high level of homoplasy resulting from the large values of haplotypic diversity and autoapomorphies translated to a low consistency index (CI = 0.384). The most obvious features of the gene-tree is that *S. sarda* haplotypes can be assigned to one of two highly divergent and extremely well supported clades, hereafter referred as Clade I and Clade II. Eighteen fixed differences comprising two indels, nine transitions and seven transversions distinguished these two clades. One synapomorphy of the Clade II haplotypes is the presence of the indel pentamer 5′-RYACA-3′ (R purine, A or G; Y Pyrimidine, C or T) at 5′-end of the light strand of the CR. The two clades are separated by D_A value of $8.1\% \pm 1.3$. The origin of these two Atlantic bonito clades appear to be monophyletic relative to the outgroup (Fig. 1), however, no conclusions about their relationship can be reached in the absence of Atlantic samples of Atlantic bonito as well as of specimens of the congeneric *S. chiliensis*.

Clade I includes 60 haplotypes belonging to 86 individuals collected in all localities sampled with a haplotypic diversity of $h = 0.985 \pm 0.006$. This clade has 70 segregating sites and a value nucleotide diversity of $\pi = 0.029 \pm 0.014$. Thus, the average difference between a random pair of Clade I fish is about 12 changes. The tree topology of Clade I is well structured with 14 nodes being supported by at least one synapomorphy resulting in those branches receiving bootstrap values >50%. Clade II included 68 haplotypes among 109 individuals, and was remarkably similar to Clade I in the number of segregating sites ($K = 71$) and in the value of haplotypic diversity ($h = 0.986 \pm 0.004$). However, the nucleotide diversity in Clade II ($\pi = 0.016 \pm 0.008$) is significantly lower (t Student, $p < 0.05$) than that in Clade I. Such disparity translates into striking differences in the branching pattern of each clade, with Clade II haplotypes characterized by star-like branching topology (Fig. 2). Accordingly, there is large uncertainty regarding the relationship of haplotypes within the sub-tree of Clade II.

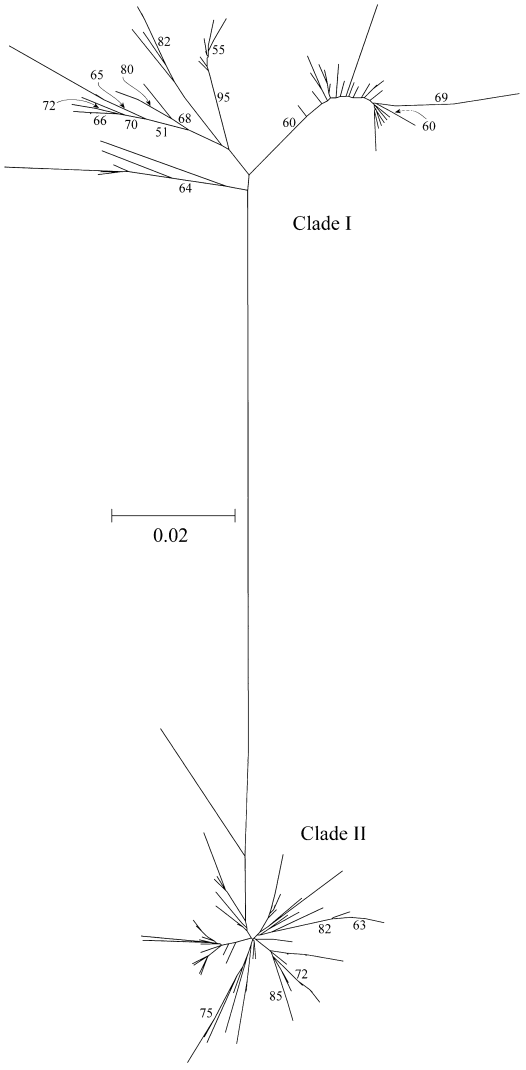


Fig. 2. Unrooted neighbor-joining (NJ) tree of the 128 Atlantic bonito haplotypes using HKY (Hasegawa et al., 1985) model of substitution with $\alpha = 0.813, i = 0.434$, transition/transversion ratio (ti/tv) of 2.456. Bootstrap values above 50% is shown by the nodes.

The mismatch distribution for the entire sample deviated significantly from the expected distribution under the sudden expansion model (Fig. 3). This outcome is supported by lack of significance of the Tajima's D test. However, independent demographic histories for each clade are suggested by the mismatch distributions and the Tajima's D of neutrality. In the case of Clade I, the bimodal distribution and a non-significant value of Tajima's D rejects the hypothesis of population bottleneck. In contrast, the mismatch distribution of Clade II together with rejection of neutrality (Fig. 3), and the star-like phylogeny (Fig. 2) suggest a recent demographic expansion.

The distribution of the two clades was highly heterogeneous among the four localities sampled (χ^2, $p = 0.001 \pm 0.001$). An east-west cline was observed, with Clade I decreasing in abundance towards the east-

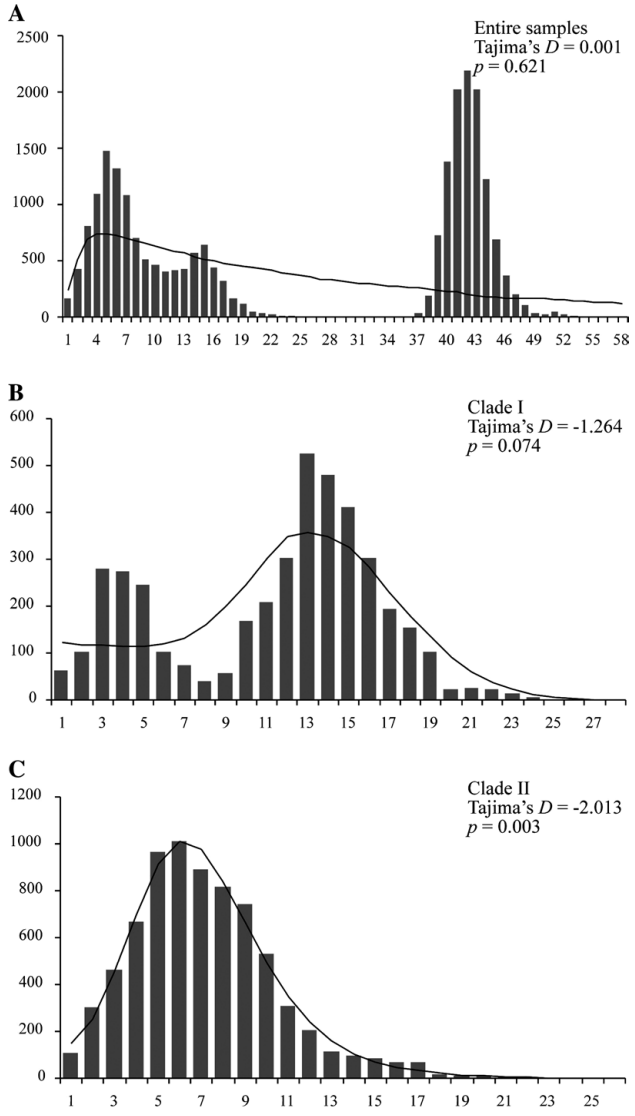


Fig. 3. Mismatch distributions and values of Tajima's D for the entire sample, Clade I and Clade II. Solid bars represent the observed pairwise differences and line the expected distribution under the sudden expansion model.

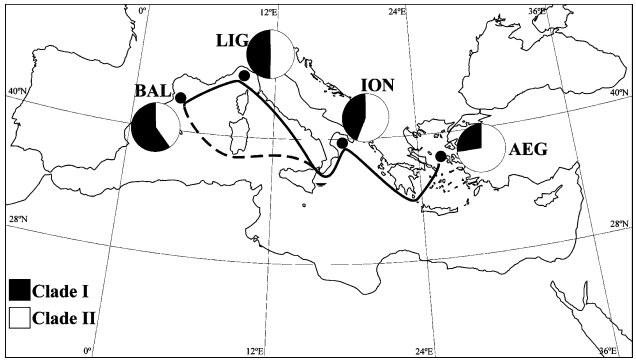


Fig. 4. Sampling locations and pie diagrams of distribution of the two mitochondrial control region clades. Lines represent the putative gene flow corridor among localities with no significant Φ_{st}. Dotted line represents the putative gene flow between the Balearic Sea and Ionian Sea. Localities abbreviations are defined in Fig. 1 legend.

Table 3
Probabilities associated to pairwise comparison of Clade I frequency among localities using Monte Carlo simulations

	Balearic Sea	Ligurian Sea	Ionian Sea
Balearic Sea			
Ligurian Sea	0.252		
Ionian Sea	0.123	0.482	
Aegean Sea	0.000[a]	0.000[a]	0.057

[a] Significant values after Bonferroni correction for multiple testing.

Table 4
Analysis of the molecular variance (AMOVA) grouping the four localities in a single group

	Variance component	% of total	Fixation index	p[a]
Among regions	1.349	6.85	Φ_{st} 0.068	0.0
Within regions	18.968	93.15		

[a] Probability of finding a more extreme variance component and Φ_{st} index than the observed by chance alone after 1000 permutations.

ern locations (Fig. 4). The highest frequency of Clade I was in the Balearic at 59.3% (32 out of 54) dropping to 50% (29 of 58) in the Ligurian and then 43.3% (13 out of 30) in the Ionian, ending with the lowest frequency, 22.6% (12 out of 53), in the Aegean. Thus, significant differences in pairwise frequency were limited to the comparisons between Aegean and Balearic samples and between Aegean and Ligurian samples (Table 3).

An AMOVA including all four localities revealed geographic heterogeneity in the distribution of genetic variation (Table 4). Although the majority (93.15%) of the genetic variance was found within locations, the amount distributed among locations (6.85%) was highly significant ($p = 0.000$). Pairwise Φ_{st} between locations and their p values are listed in Table 5. The two western Mediterranean localities, the Balearic and the Ligurian, differed significantly from the Aegean Sea—the most eastern locality sampled. The Ionian Sea sample was not different from any other sample; a result concordant with expectations for intermediate locations in an IBD model. Estimates of the number of migrants per generation (N_m) derived from Φ_{st} values (Slatkin, 1991) is very low between the two most remote locations ($N_m = 2.133$; Table 5) but increases towards infinity in the comparison between the Ligurian Sea and Ionian Sea samples.

Fig. 4 summarizes the putative corridors of gene flow among the locations. The connection lines represent the shortest geographic distance connecting each adjacent pair of samples whose Φ_{st}s were not significantly different. The resulting pattern includes a genetic corridor via northern Mediterranean coastal waters which connects all four locations. Additionally, the lack of differentiation between the Balearic Sea and the Ionian Sea

Table 5
Genetic differentiation matrix of localities stated by $\Phi_{st}s$ and p values in parenthesis (below diagonal)

	Balearic Sea	Ligurian Sea	Ionian Sea	Aegean Sea
Balearic Sea		505.687	14.682	2.133
Ligurian Sea	0.001 (0.268)		Inf	4.001
Ionian Sea	0.033 (0.097)	−0.003 (0.364)		8.316
Aegean Sea	0.189 (0.000[a])	0.111 (0.000[a])	0.056 (0.055)	

Number of migrants per generation estimated from the pairwise $\Phi_{st}s$ values (above diagonal).
[a] Significant values after Bonferroni correction for multiple testing.

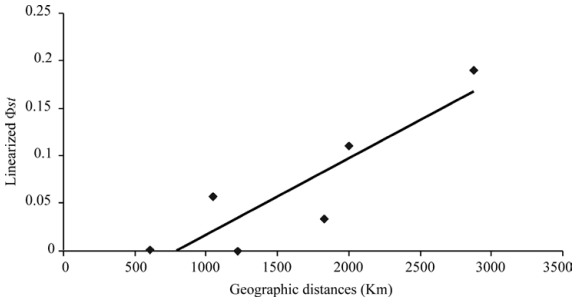


Fig. 5. Correlation between geographic distance and pairwise linearized Φ_{st}s. Regression equation and coefficient are: $y = 8 \times 10^{-5}x - 0.0632$; $r^2 = 0.780$.

samples suggests direct contact (historical or ongoing) between these two locations through the waters south of Sardinia (dotted line in Fig. 4). A Mantel test revealed a significant correlation between Φ_{st}s and geographic distances ($p = 0.032$) associated to these putative gene flow corridors, one which involves the alternative contact between Balearic Sea and Ionian Sea ($p = 0.036$). The correlation between Φ_{st} and geographic distance was confirmed by the positive slope of the first order regression line which is significantly different from zero ($p = 0.003$) for the northern shortest path (Fig. 5). It is relevant to note that once again the direct gene flow between the Balearic and the Ionian, as interpreted from the significant regression test ($p = 0.003$), cannot be rejected.

4. Discussion

4.1. Patterns of variation of mtDNA control region in Atlantic bonito

The mtDNA CR for Atlantic bonito is extremely variable. The DNA sequence analysis of the CR from 195 Atlantic bonito revealed that more than 25% of the nucleotide positions were variable, whereas in Cyt*b* less of 5% of the nucleotide position were polymorphic (Roberti et al., 1993). Extremely high values of haplotypic diversity were found in the four geographic locations sampled with the majority of haplotypes sampled only once in most localities. The exception was the Aegean sample where the frequency of repeated haplotypes was much higher with about 43% of the haplotypes sampled at least twice. Interestingly, such disparity in the Aegean is irrespective of the phylogenetic origin of the repeated haplotypes, which is indicative of a unique set of demographic forces that have affected Atlantic bonito mtDNA in that region. Consequently, it could be predicted that the female effective population size (N_{ef}) in the Aegean is lower than in any other locality surveyed in this study. Larger sample sizes and both mitochondrial and nuclear (e.g., microsatellite loci) would be desirable to provide good estimates of N_e of this species in the Mediterranean. In general diversity values are high, and the nucleotide diversity value estimated for the mtDNA CR Atlantic bonito in the Mediterranean ($\pi = 0.063 \pm 0.031$) ranks among the highest reported among member of the family Scombridae including *Thunnus* species ($\pi = 0.017$–0.072, Alvarado Bremer et al., 1997), Atlantic mackerel (*Scomber scombrus*) ($\pi = 0.029$; Nesbø et al., 2000), albacore tuna (*Thunnus alalunga*) ($\pi = 0.054$, Viñas et al., 2004), and little tunny (*Euthynnus alletteratus*) ($\pi = 0.057$; Alvarado Bremer and Ely, 1999). Such an extreme value of π in Atlantic bonito could be an artifact of the comparison of two sets of highly divergent (8.1%) lineages that originated allopatrically. Both the mismatch distribution analysis and the east-west cline which characterize the distribution of these two clades supports vicariance followed by secondary contact (see below). If this interpretation is correct, a more accurate approach to assess levels of variability would to estimate the π values separately for Clade I ($p = 0.029$) and Clade II, ($\pi = 0.016$). These values are comparable to the levels of mtDNA CR variability reported in other scombroid fishes.

4.2. Do separate clades correspond to cryptic species?

European anchovy (*Engraulis encrasicolus*) depicts a strikingly similar pattern of differentiation of mtDNA to Atlantic bonito described here (Magoulas et al., 1996). This pattern corresponds to a species complex co-existing within the Mediterranean (Borsa, 2002). It should be noted that the D_A value in Atlantic bonito from the Mediterranean is larger than any reported for the orthologous mtDNA segment in Scombridae species (Table 6). Accordingly, the possibility exists that one these clades correspond to a separate cryptic species. Fur-

Table 6
Genetic divergence (D_A) between clades in several Scombridae species using the mtDNA CR

Common name	Species name	D_A (%)	References
Atlantic bonito	*Sarda sarda*	8.1	This study
Bigeye tuna	*Thunnus obesus*	5	Alvarado Bremer et al., 1998
Albacore tuna	*T. alalunga*	3.3	Viñas et al., 2004
Atlantic mackerel	*Scomber scombrus*	4.7	Nesbø et al., 2000

thermore, the inter-clade divergence value reported here for Atlantic bonito is larger than the divergence values between tuna species of the subgenus *Neothunnnus* (5.9–7.9%) which includes yellowfin tuna (*Thunnus albacares*), longtail tuna (*T. tonggol*), and blackfin tuna (*T. atlanticus*) (Alvarado Bremer et al., 1997). However, the inter-clade divergence in Atlantic bonito is considerably smaller than that obtained from the binary comparisons of other *Sarda* species such as Atlantic bonito and striped bonito ($D_A = 16.1\% \pm 1.1$), Atlantic bonito and Australian bonito ($D_A = 12.9\% \pm 1.8$) and striped bonito and Australian bonito ($D_A = 11.7\% \pm 1.9$) (this study). Thus, values of inter-species divergence in *Sarda* are about two times higher than within *Neothunnus* but much smaller than the distance separating *Sarda* species. Since the rate of molecular evolution is a function of generation time (Li and Tanimura, 1987) the disparity reported here can be accounted by the two to threefold shorter generation times of bonitos compared to tunas. Furthermore, Pujolar et al. (2001) reported no deviation from Hardy–Weinberg equilibrium within the Ligurian, Ionian, or Aegean Sea samples of Atlantic bonito. Accordingly, neither the magnitude of the divergence value reported here between Atlantic Bonito clades, nor the absence of departures from HW expectations from the allozyme data of Pujolar et al. (2001) warrant the presence in the Mediterranean of a cryptic species as suggested for European anchovy by Borsa (2002).

4.3. Population structure

The null hypothesis of panmixia for Atlantic bonito in the Mediterranean can be rejected based upon the heterogeneous geographic distribution of two mitochondrial clades. The genetic heterogeneity within the Mediterranean is also supported by a small ($\Phi_{st} = 6.8\%$) but highly significant ($p = 0.000$) portion of the genetic variance. A major difficulty associated with the low F_{st} values characteristic of highly migratory oceanic species is the ability to discriminate between low levels of population differentiation and artifacts due to noise related to sampling error (Waples, 1998). In the present study, the asymmetric distribution of the two mitochondrial clades along the Mediterranean (Fig. 4) suggests that IBD played a determinant role in modeling the population structure of Atlantic bonito in that basin, as evidenced by positive correlation of genetic differentiation and geographic distance from east to west (Fig. 5). This pattern is more likely due to limited gene flow among geographically distant populations as opposed to sampling noise (Palumbi, 2003). Under this scenario, the pairwise comparison of Φ_{st} values can be interpreted as gene flow corridors through the coastal waters of the north Mediterranean Sea connecting all four Mediterranean locations surveyed. Gene flow levels between the Aegean and eastern locations occurrs at low levels (Table 5). The estimated level of gene flow of about four individuals per generation (i.e., number of migrants between the Ligurian and Aegean locations, Table 5) and of about two individuals per generation (i.e., between the two extremes of the range, Table 5) is insufficient to cause the genetic homogenization of mtDNA in the Mediterranean. These levels of migration are consistent with the expectation of at least four individuals per generation are needed to homogenize subpopulations with same migration rates of males and females (Birky et al., 1983).

The significant correlation of the genetic differentiation and geographic distance contrasts with the conclusion reached in previous studies using Cyt*b* data (Roberti et al., 1993) and allozyme data (Pujolar et al., 2001). In these studies, genetic differentiation is explained as a function of a putative barrier to gene flow that limits the contact between Atlantic bonito populations inhabiting the eastern and the western Mediterranean. Given that a sample from the Sea of Marmara was not included in this study, the possible presence of a barrier to gene flow separating that basin from the Aegean as suggested by Roberti et al. (1993) cannot be discarded. However, the IBD interpretation presented here suggests gene flow decreases with increasing geographical distance. Thus, no barriers to gene flow are necessary to explain genetic heterogeneity. Both the extended coverage and the use phylogeography, as opposed to the reliance on the interpretation of F_{st} statistic alone, gave us the opportunity to offer alternative mechanisms to explain the heterogeneous distribution of clades in an east-west cline within the Mediterranean Sea. Interestingly, a close inspection of the Cyt*b* data of Roberti et al. (1993) reveals the presence of two sets of sequences that shift in relative frequency along the region sampled, a result concordant with the IBD reported here. Similarly, in the study of Pujolar et al. (2001) the unlinked loci Esterase (*EST**), Lactate de-

hydrogenase (*LDH-1**), and mannose-6-phosphate isomerase (*MPI**) depict an east-west cline. Interestingly, *EST** and *MPI** are two of the three loci that showed significant differentiation in allele frequencies between the Aegean Sea relative to the Ligurian Sea and the Ionian Sea, and which were used by Pujolar et al. (2001) to place a barrier to gene flow separating the putative eastern and western populations.

The significant association between gene flow and geographic distance observed in a vagile species such as the Atlantic bonito within a relatively small region was unexpected. Examples of IBD in marine species with large dispersal capabilities have been typically documented over large geographic areas (Palumbi, 1994). The observed pattern of population structure is surprising, particularly when compared to other Scombridae species with similar neritic distribution. In Atlantic mackerel (*Scomber scombrus*), Nesbø et al. (2000) detected IBD only when comparing the most distant samples, namely Canadian Atlantic coast and the Adriatic Sea. In chub mackerel (*Scomber japonicus*), the distribution of mtDNA haplotypes among collections from the Mediterranean Sea, Ivory Coast, and South Africa was not heterogeneous (Graves, 1998). Furthermore, both Spanish mackerel (*Scomberomorus maculatus*) and king mackarel (*S. cavalla*) showed no genetic differentiation in the comparison of samples from the Gulf of Mexico and US Atlantic coast (Buonaccorsi et al., 2001; Gold and Richardson, 1998) separated by a geographic distance comparable to that between the two most extreme geographic locations surveyed in this study. Furthermore, Gold and Richardson (1998) reviewed studies of mitochondrial variation in several fishes distributed along the northwestern Atlantic and the Gulf of Mexico. Population subdivision, following IBD, was only documented in species that are estuarine-dependent during the larval and juvenile stages. Since Atlantic bonito displays no association to estuaries during its lifetime, other mechanisms must be involved to maintain genetic differentiation in this vagile species.

Two alternative mechanisms can be invoked to explain the presence of genetic heterogeneity in Atlantic bonito at the small spatial scales of the Mediterranean Sea. First, larval retention (Sinclair and Iles, 1985) could play an important role in establish genetic heterogeneity in species with pelagic larvae (Gold and Richardson, 1998; Taylor and Hellberg, 2003). The patterns of surface current in the Mediterranean Sea could limit larval dispersal, provided that adults do not disperse among regions. Such influence has been invoked by Magoulas et al. (1996) to account the observed patterns of differentiation in European anchovy, particularly with regard to the distinctiveness of the Aegean Sea and the Adriatic Sea sub-populations. Second, adult individuals may observe a natal philopatric behavior towards respective spawning grounds. Atlantic bonito in the Mediterranean observe annual migratory movement between feeding and spawning grounds independently in the east and west Mediterranean. One group is known to spawn in the early summer in the western Mediterranean between Gibraltar, Balearic Islands, and Algeria. After spawning, individuals return to the feeding areas, which include Atlantic waters adjacent to the Strait of Gibraltar, and the Spanish and Moroccan Mediterranean coasts (Rey et al., 1984). At about the same time, a very similar pattern of migration has been described in the eastern Mediterranean Sea connecting the feeding grounds of the Aegean Sea and the spawning areas of the Sea of Marmara with Black Sea (Yoshida, 1980). The clinal pattern of variation documented in this study may suggests that each of the locations sampled represents one smaller deme with some gene flow between them leading to the observed genetic differentiation gradient. The recent characterization of a new reproductive area in north of the Balearic Islands (Sabatés and Recasens, 2001) supports the possible presence of additional spawning areas along the Mediterranean coast.

4.4. Phylogeographic origin of genetic mitochondrial discontinuity

The presence of highly divergent broadly sympatric mtDNA clades within species (category II; Avise, 2000) has been interpreted as evidence of vicariance followed by reinvasion in highly migratory species such as bigeye tuna, blue marlin, sailfish, and swordfish (Alvarado Bremer et al., 1995; Alvarado Bremer et al., 1996; Alvarado Bremer et al., 1998; Finnerty and Block, 1992; Graves, 1998; Graves and Mcdowell, 1995). It can be argued that one possible way to account for the absence of intermediate lineages in an apparent genetic discontinuity is the omission of sampling of geographically intermediate locations (Templeton, 1998) where intermediate lineages may be found. However, the longitudinal sampling of the Mediterranean conducted in this study was adequate and no intermediate haplotypes were found. Alternatively, phylogenetic discontinuities can be caused by demographic factors as predicted from expectations of large stable populations under the neutral coalescence theory (Harpending et al., 1998; Slatkin and Hudson, 1991). Recently, Nesbø et al. (2000) and Viñas et al. (2004) invoked this model to explain the topology of the mtDNA CR tree in Atlantic mackerel and albacore tuna, respectively. For Atlantic bonito in the Mediterranean the bimodal mismatch distribution of the entire data set and the acceptance of neutrality (Fig. 3A) suggest the presence of a large stable population for Atlantic bonito in the Mediterranean. A detailed inspection of the mismatch distribution, respectively, for each clade reveals a bimodal distribution for Clade I (Fig. 3B), consistent with a large stable population, particularly since there is no phylogenetic

association of groups of lineages (Fig. 1), and also supported by the non-significant Tajima's *D* value. However, for Clade II there is a close correspondence between observed and predicted mismatch distributions, as corroborated by the significant negative values of Tajima's *D* test (Fig. 3C) and the star-like phylogeny of this clade (Fig. 2). This implies that in the recent past a portion of the Atlantic bonito population experienced a bottleneck followed by a sudden population expansion. Consequently, the bimodal mismatch distribution obtained from the combined analysis of both clades should be not be interpreted as evidence of a large stable population for this species. Star-like phylogenies can also result from the selective sweep of a phenotypically advantageous allele followed by accumulation of neutral variants (Maruyama and Birky, 1991). A selective sweep, at one locus reduces variation at linked loci, but not at unlinked loci (Brookfield, 2001). However, in Atlantic bonito low levels of genetic variation were also observed at most allozyme loci (Pujolar et al., 2001). Thus, it seems unlikely that selection would affect the variation of unlinked mtDNA and nuclear loci at the same time.

Alvarado Bremer et al. (1995) associated the division of two clades in swordfish mtDNA CR to the isolation of the Mediterranean and Atlantic populations due to drop in sea level caused by Pleistocene eustatic events. In Atlantic bonito in the Mediterranean, the increase in frequency of Clade I fish towards the Atlantic may indicate that this clade originated in that ocean basin. Evidence of ongoing migratory movement across Gibraltar (Rey and Cort, 1981) supports that notion. If this interpretation is correct, Atlantic fish should have haplotypes belonging primarily or exclusively to Clade I. The morphometric analysis of western Atlantic and Mediterranean samples indicates potential differences between these two populations (Collette and Chao, 1975) which may coincide with the documented genetic differences. Currently, samples from these areas are being procured as part of more extensive study of Atlantic bonito.

The results of this study can be summarized as follows. Two very distinct clades in Atlantic bonito originated in vicariance during the Pleistocene. Clade II suffered a substantial population collapse, followed by sudden expansion. The observed east-west cline is the result of secondary contact of formerly allopatric populations, and the observed pattern of differentiation along this gradient is maintained by IBD.

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