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Microsatellite and mitochondrial DNA analyses of Atlantic bluefin tuna (*Thunnus thynnus thynnus*) population structure in the Mediterranean Sea

JENS CARLSSON,* JAN R. McDOWELL,* PÍNDARO DÍAZ-JAIMES,*† JEANETTE E. L. CARLSSON,* SANDRA B. BOLES,* JOHN R. GOLD‡ and JOHN E. GRAVES*

**Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Rt. 1208, Greate Road, Gloucester Point, VA 23062-1346, USA; †Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Apartado Postal 70-305, México D.F. 04510, México; ‡Center for Biosystematics and Biodiversity, Department of Wildlife and Fisheries, Texas A & M University, College Station, TX 77843-2258, USA*

Abstract

Genetic variation was surveyed at nine microsatellite loci and the mitochondrial control region (868 bp) to test for the presence of genetic stock structure in young-of-the-year Atlantic bluefin tuna (*Thunnus thynnus thynnus*) from the Mediterranean Sea. Bluefin tuna were sampled over a period of 5 years from the Balearic and Tyrrhenian seas in the western basin of the Mediterranean Sea, and from the southern Ionian Sea in the eastern basin of the Mediterranean Sea. Analyses of multilocus microsatellite genotypes and mitochondrial control region sequences revealed no significant heterogeneity among collections taken from the same location in different years; however, significant spatial genetic heterogeneity was observed across all samples for both microsatellite markers and mitochondrial control region sequences (F_{ST} = 0.0023, P = 0.038 and Φ_{ST} = 0.0233, P = 0.000, respectively). Significant genetic differentiation between the Tyrrhenian and Ionian collections was found for both microsatellite and mitochondrial markers (F_{ST} = 0.0087, P = 0.015 and Φ_{ST} = 0.0367, P = 0.030, respectively). These results suggest the possibility of a genetically discrete population in the eastern basin of the Mediterranean Sea.

Keywords: D-loop, highly migratory, microsatellites, population genetic, spawning area, teleost

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Introduction

Highly migratory marine teleosts often have cosmopolitan distributions and occur throughout large areas of the world's oceans. The highly migratory ability of these fishes, combined with the marine environment's lack of pronounced barriers to gene flow, are thought to preclude the development of a strong signal of population genetic structure (Waples 1998; Smedbol *et al*. 2002). Analysis of the population structure of highly migratory species is further complicated by unique sampling considerations. Because individuals are capable of making extensive migrations, there is uncertainty regarding the natal origin of all but the youngest life history stages (Graves *et al*. 1996). These factors make studies of highly migratory species, including the North Atlantic bluefin tuna (*Thunnus thynnus thynnus*), a special challenge for population geneticists.

Increased fishing effort has lead to a global reduction in abundances of large predatory fishes, and the Atlantic bluefin tuna is no exception (Myers & Worm 2003). In the Atlantic Ocean, bluefin tuna are severely overfished (NMFS 1995), and are considered to be the most threatened of the tuna species (Magnuson *et al*. 1994). The International Commission for the Conservation of Atlantic Tunas (ICCAT) has managed Atlantic bluefin tuna as separate western and eastern stocks since 1982. The presence of two stocks was supported by discontinuities in catches across the North Atlantic and the observation that spawning is known to occur in only two areas, the Mediterranean Sea in the east and the Gulf of Mexico in the west (Mather *et al*. 1995). However, over the past 30 years, considerable information has accrued which has caused scientists and

Correspondence: Jens Carlsson, Fax: +1 804 6847157; E-mail: jc@vims.edu

managers to question the validity of the two-stock model (Magnuson *et al*. 1994). For example, it is known that bluefin tuna occur across the Atlantic Ocean, and a fishery has developed in the central Atlantic. Furthermore, conventional and electronic tagging studies have demonstrated that some individuals undertake transatlantic movements (Mather *et al*. 1995; Block *et al*. 2001).

The need to elucidate appropriate management units for Atlantic bluefin tuna has led to several genetic studies employing a range of molecular markers. Previous studies of the Atlantic-wide genetic population structure of bluefin tuna based on allozyme variability have not detected any population structure (Edmunds & Sammons 1973; Thompson & Contin 1979; Pujolar *et al*. 2003). Pujolar *et al*. (2003) did detect significant Atlantic-wide differentiation at one allozyme locus, but these differences disappeared when using multilocus estimates. Broughton & Gold (1997) used microsatellite analyses on collections from the western and eastern Atlantic and found small but significant Atlantic-wide population structure. Takagi *et al*. (1999) employed microsatellites on collections of bluefin tuna from the eastern and western Atlantic and found no Atlantic-wide structure. Alvarado Bremer *et al*. (1999) sequenced the mitochondrial (mtDNA) control region of bluefin tuna from the western and eastern Atlantic and found significant differentiation between the two collections. However, the authors of both microsatellite studies (Broughton & Gold 1997; Takagi *et al*. 1999) and the mtDNA study by Alvarado Bremer *et al*. (1999) were reluctant to draw explicit conclusions about the population structure of bluefin tuna in the Atlantic because of the low sample sizes included in their studies. Ely *et al*. (2002) found no indication of Atlantic-wide population structure when using either sequences of the mtDNA control region or restriction fragment length polymorphism (RFLP) variation at the nuclear encoded *ldhA* gene on collections from the western Atlantic and the Mediterranean Sea. These studies have resulted in different conclusions regarding the population structure of bluefin tuna and, hence, there has been no consensus on the Atlantic-wide stock structure of bluefin tuna. Less effort has been made to investigate population structuring on more regional scales.

The Mediterranean Sea has supported a bluefin tuna fishery throughout recorded history (Maggio 2000), and, owing to increased fishing effort, the Mediterranean Sea now supports the world's largest bluefin tuna fishery (Restrepo *et al*. 2003). Bluefin tuna are known to spawn at two locations in the western Mediterranean Sea, off the Balearic Islands and in the southern Tyrrhenian Sea (Dicenta 1977; García *et al*. 2001; Medina *et al*. 2002). In addition, mature bluefin tuna are known to occur in the eastern Mediterranean Sea, and studies by Karakulak *et al*. (2004) concluded that there may be spawning in this area as well.

The Mediterranean Sea is a semi-enclosed system consisting of two partly isolated basins (western and eastern) connected by the Strait of Sicily and the narrow Strait of Messina (Robinson 2001). The two basins differ in their oceanography, particularly their thermal structures, salinity profiles, and circulation regimes (Millot 1999; Robinson 2001). Population genetic studies of sand goby, *Pomatoschistus minutus* (Stefanni & Thorley 2003), Mediterranean sea bass, *Dicentrarchus labrax* (Bahri-Sfar *et al*. 2000) and poor cod, *Trisopterus minutus capelanus* (Mattiangeli *et al*. 2003) have shown population genetic structuring between conspecific collections from the two basins. Genetic heterogeneity within the Mediterranean Sea was also found among collections of bonito, *Sarda sarda* (Pujolar *et al*. 2001). In contrast, swordfish, *Xiphias gladius*, showed no genetic population structuring within the Mediterranean Sea using either restriction fragment length polymorphism (RFLP) analysis of mtDNA (Kotoulas *et al*. 1995) or allozyme analyses (Pujolar *et al*. 2002). Similarly, allozyme analyses did not reveal any intra-Mediterranean Sea population structure in albacore, *Thunnus alalunga* (Pujolar *et al*. 2003). Previous studies of bluefin tuna genetic population structure within the Mediterranean Sea are equivocal. Broughton & Gold (1997), using five microsatellite loci, found significant heterogeneity among bluefin tuna samples from the western and eastern basins of the Mediterranean Sea, although the small sample sizes ($n = 8$ and $n = 12$) precluded the authors from making firm conclusions. Alvarado Bremer *et al*. (1999) used mtDNA control region sequences of bluefin tuna from different locations in the Mediterranean Sea and found no geographical heterogeneity among the collections. Viñas *et al*. (2003) sequenced the mtDNA control region of bluefin tuna from three different localities in the Mediterranean Sea, which resulted in detection of significant genetic heterogeneity among samples. However, they (Viñas *et al*. 2003) suggested that the low sample size ($n = 12$) of one of their collections probably was responsible for the significant outcome of the analysis. Pujolar *et al*. (2003) found no evidence of population structure among 16 collections of bluefin tuna within the Mediterranean Sea based on allozyme markers. The lack of consensus regarding the population structure of bluefin tuna in the Mediterranean Sea, combined with the presence of potentially discrete western and eastern Mediterranean Sea basin bluefin tuna spawning areas suggests the possibility of stock structure and underscores the need for population genetic studies of bluefin tuna within the Mediterranean Sea.

We screened 280 bluefin tuna for genetic variability at nine microsatellite loci and sequenced the mitochondrial control region in a subset of 74 bluefin tuna to test for population structure among bluefin tuna within the Mediterranean Sea. Temporal collections of young-of-the-year (YOY) bluefin tuna from the Balearic and Tyrrhenian seas

in the western basin of the Mediterranean Sea and the Ionian Sea in the eastern basin of the Mediterranean Sea were analysed to test the null hypothesis of no population structure among bluefin tuna from the western and eastern basins.

Materials and methods

Biological materials

Muscle tissue samples, collected from bluefin tuna caught by commercial vessels, were acquired from a total of 280 YOY bluefin tuna at three locations in the Mediterranean Sea over a period of 5 years. These included the Balearic Sea in 1998 (Ba98) and 1999 (Ba99), the Tyrrhenian Sea in 1998 (Ty98), 1999 (Ty99), and 2002 (Ty02), and the Ionian Sea in 1998 (Io98) and 1999 (Io99) (Fig. 1, Table 1). Muscle tissue was stored in buffer (saturated NaCl, 250 mM ethylenediaminetetraacetic acid (EDTA), 20% dimethyl sulphoxide (DMSO)) and kept at room temperature until DNA was isolated.

DNA analysis

Total genomic DNA was isolated using either a phenol–chloroform (Sambrook *et al*. 1989) or proteinase K–chelex extraction (Estoup *et al*. 1996). Nine microsatellite loci were amplified (more detailed information about polymerase chain reaction (PCR) conditions will be provided upon request) and analysed: *Tth5*, *Tth8*, *Tth10*, *Tth21* and *Tth34* (McDowell *et al*. 2002); and *Ttho-1*, *Ttho-4*, *Ttho-6* and *Ttho-7* (Takagi *et al*. 1999). Microsatellite polymorphism was analysed on a Li-Cor 4200 Global IR² automated sequencer (Li-Cor, Lincoln, NE, USA). A 50–350 bp sizing standard (Li-Cor) was run at the centre and at both extremes of the gel to determine allele size. At least four lanes in each run consisted of individuals for which the allele sizes were known to ensure identical allele scoring across runs. In addition, approximately 20% of the samples were run again to confirm repeatability of allele scoring. Fragment length polymorphism was analysed with the GENE IMAGIR software (Li-Cor).

The mtDNA control region (D-loop, 868 bp), was amplified with the primers Pro-5′-and 12SAR-3′ (Palumbi 1996; CAC GAC GTT GTA AAA CGA CCT ACC YCY AAC TCC CAA AGC and GGA TAA CAA TTT CAC ACA GGG CAT AGT GGG GTA TCT AAT CC), respectively. Primers were modified to include degeneracies and M13 tails (K. Gray, Virginia Institute of Marine Sciences, unpublished). The resulting product was sequenced for a subset of individuals from each collection. A total of 74 individuals were amplified: 25 bluefin tuna from the Ba (13 Ba98 and 12 Ba99), 24 from the Io (8 Io98 and 16 Io99), and 25 bluefin tuna from the Ty collections (9 Ty98, 8 Ty99 and 8 Ty02). Amplified products were sequenced using IRD-800-labelled forward primer and IRD-700-labelled reverse primer (Li-Cor) on a Li-Cor 4200 Global IR² system. Sequencing primers were M13F(-29) and an internal reverse primer designed specifically for teleost control region (CCA TCT TAA CAT CTT CAG TG; S. B. Boles, Virginia Institute of Marine Sciences, unpublished). In many cases, single-stranded sequencing with the internal reverse primer yielded unambiguous sequence. The forward sequence was generated when necessary to resolve ambiguities. Standard chromatographic curves of forward and reverse sequences were imported into the program SEQUENCHER version 3.0 (Gene Codes Corp., Ann Arbor, MI, USA), aligned and edited. Consensus sequences were exported to the program MAC VECTOR version 6.5 (Oxford Molecular Ltd, Madison, WI, USA), aligned with other sequences using the CLUSTALW algorithm (Thompson *et al*. 1994), and adjusted by eye.

Statistical analyses

The software MICRO-CHECKER 2.2.1 (van Oosterhout *et al*. 2004) was used for identifying possible genotyping errors (i.e. stuttering, large allele dropout and null-alleles) within

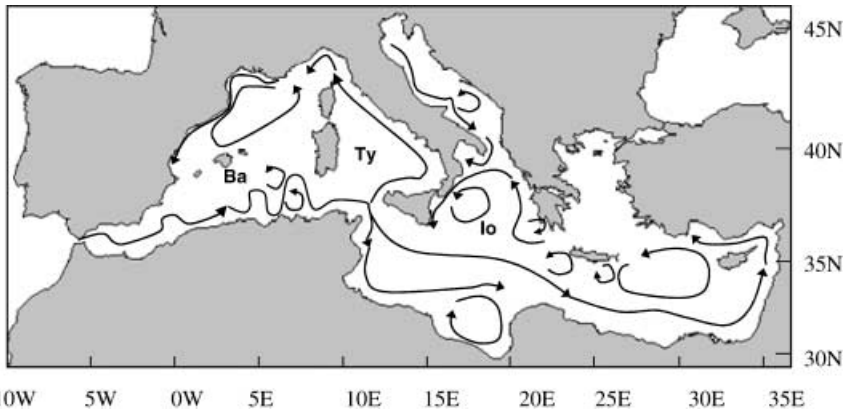

Fig. 1 Locations of bluefin tuna samples in the Mediterranean Sea. Ba, Balearic Sea; Ty, Tyrrhenian Sea; Io, Ionian Sea; arrows indicate dominating surface currents. Redrawn from Pinardi & Masetti (2000).

Table 1 Summary statistics for nine microsatellite loci among bluefin tuna collections

Sample	Locus									Average across loci
	Tth5	Tth8	Tth10	Tth21	Tth34	Ttho-1	Ttho-4	Ttho-6	Ttho-7	
Ba98										
n	74	74	74	74	74	74	74	70	74	
a	5	13	3	3	16	6	10	18	15	9.89
Rs	2.94	6.26	2.12	2.32	7.67	3.85	5.94	9.09	8.11	5.37
as	125–181	294–346	118–124	125–133	105–185	181–195	128–166	127–189	180–224	
H_E	0.506	0.785	**0.441**	0.459	0.825	0.609	0.760	0.892	0.862	0.68
H_O	0.500	0.784	0.608	0.405	0.757	0.581	0.703	**0.557**	0.878	0.64
HW	0.776	0.462	**0.002**	0.559	0.024	0.549	0.388	**0.000**	0.897	
Ba99										
n	60	60	60	60	60	60	60	60	60	
a	4	12	2	3	16	7	12	19	14	9.89
Rs	2.56	6.11	2.00	2.28	7.37	3.86	7.04	9.80	7.74	5.42
as	125–133	196–346	118–122	117–133	101–165	181–197	136–168	127–247	180–224	
H_E	0.505	0.763	0.464	0.459	0.795	0.604	0.823	0.910	0.823	0.68
H_O	0.450	0.650	0.517	0.533	0.883	0.650	0.817	**0.633**	0.783	0.66
HW	0.039	0.023	0.410	0.314	0.791	0.946	0.252	**0.000**	0.116	
Ty98										
n	28	28	28	28	28	28	28	28	28	
a	5	10	2	3	16	5	10	14	10	8.33
Rs	3.18	6.79	1.99	2.32	7.64	4.07	6.61	7.80	7.62	5.34
as	125–145	296–342	118–122	125–133	101–165	181–195	128–166	123–229	180–220	
H_E	0.468	0.821	0.321	0.441	0.772	0.665	0.793	0.805	0.871	0.66
H_O	0.464	0.893	0.393	0.500	0.893	0.643	0.821	0.643	0.929	0.69
HW	0.101	0.372	0.547	0.302	0.777	0.255	0.313	0.086	0.829	
Ty99										
n	33	33	33	33	33	33	33	33	33	
a	6	10	2	3	14	5	13	14	13	8.78
Rs	3.44	6.21	2.00	2.47	7.52	3.53	6.98	8.92	8.75	5.54
as	125–189	298–342	118–122	125–133	101–165	181–195	128–188	127–185	196–222	
H_E	0.526	0.763	0.491	0.507	0.788	0.628	0.814	0.890	0.869	0.70
H_O	0.455	0.788	0.455	0.515	0.788	0.606	0.848	**0.636**	0.909	0.67
HW	0.077	0.192	0.729	1.000	0.569	0.313	0.244	**0.000**	0.847	
Ty02										
n	63	60	63	63	63	63	63	63	63	
a	5	11	2	4	15	7	13	20	15	10.22
Rs	3.18	6.29	2.00	2.81	7.77	4.03	6.85	9.31	8.41	5.63
as	125–175	298–342	118–122	117–133	101–185	181–195	128–184	127–189	180–222	
H_E	0.520	0.796	0.437	0.530	0.813	0.662	0.818	0.902	0.864	0.70
H_O	0.508	0.833	0.540	0.508	0.825	0.619	0.857	0.762	0.889	0.70
HW	0.395	0.987	0.079	0.473	0.063	0.100	0.322	0.034	0.658	
Io98										
n	9	9	9	9	9	9	9	9	9	
a	3	9	2	2	9	4	5	7	7	5.33
Rs	3.00	9.00	2.00	2.00	9.00	4.00	5.00	7.00	7.00	5.33
as	125–133	298–348	118–122	125–129	105–145	181–195	138–166	127–189	206–222	
H_E	0.392	0.882	0.529	0.529	0.895	0.314	0.784	0.804	0.850	0.66
H_O	0.444	0.889	0.778	0.556	1.000	0.222	0.778	0.556	0.778	0.67
HW	1.000	0.565	0.228	1.000	0.869	0.182	0.395	0.042	0.511	
Io99										
n	16	16	16	16	16	16	16	16	16	
a	3	7	2	2	12	7	6	13	11	7.00
Rs	2.93	5.89	2.00	2.00	8.13	5.42	4.69	10.18	8.74	5.55
as	125–133	298–342	118–122	125–129	101–165	179–195	138–166	127–193	127–189	
H_E	0.538	0.790	0.516	0.466	0.796	0.627	0.718	0.917	0.889	0.69
H_O	0.563	0.875	0.375	0.438	0.875	0.563	0.750	**0.563**	0.813	0.65
HW	1.000	0.027	0.343	1.000	0.135	0.287	0.604	**0.000**	0.181	

Ba, Balearic; Ty, Tyrrhenian; Io, Ionian; *n*, number of individuals ; *a*, number of alleles; *Rs*, allele richness per locus and sample; *as*, allele size range in base pairs; H_E, expected heterozygosity; H_O, observed heterozygosity; *HW*, probability values of concordance with Hardy–Weinberg expectations.

Values in bold type are significant probability estimates after correction for multiple tests (initial $\alpha = 0.05/9 = 0.0056$).

the microsatellite data set by performing 1000 randomizations. The GENEPOP 3.1b software package (Raymond & Rousset 1995) was used to test for deviations of genotypic distributions from Hardy–Weinberg expectations (exact tests; Guo & Thompson 1992), as well as for calculations observed (H_O) and expected (H_E) heterozygosities, and to test for heterozygosity excess and deficiency (exact tests) for microsatellite data, and for estimating global single locus F_{IS}. Further, the software FSTAT 2.9.3.2 (Goudet 1995) was used on the microsatellite data set to estimate global single locus gene diversities (H_T) and allele richness per locus and sample (*Rs*). The program ARLEQUIN (Schneider *et al*. 1997) was used to calculate haplotype diversity (*h*) and nucleotide diversity (π) for the mtDNA sequence data. The software PAUP* 4.0 (Swofford 2000) was used to generate a table of variable sites (see Electronic Appendix) and for constructing a neighbour-joining tree based on the Tamura & Nei (1993) distance (Fig. 2). In all cases with multiple tests, significance levels were adjusted using the sequential Bonferroni technique (Rice 1989).

We used DNASP 4.0 (Rozas *et al*. 2003) to estimate the nearest-neighbour statistic, S_{nn} (Hudson 2000) for the mtDNA control region sequences (10 000 permutations). This statistic is used on haplotypic data in the form of DNA sequences or many tightly linked markers and uses a symmetric island model; it also assumes an infinite-site model of mutation. S_{nn} is a measure of how often the 'nearest neighbours' (in sequence space) of sequences are from the same locality in geographical space and it is particularly suitable when haplotype diversity is large and sample sizes are small (Hudson 2000).

The ARLEQUIN software package was used to estimate (Weir & Cockerham 1984) unbiased estimator of Wrights *F*-statistics (F_{ST}) and Φ_{ST} (an mtDNA analogue for F_{ST}; Excoffier *et al*. 1992) and for hierarchical F_{ST} and Φ_{ST} analyses (10100 permutations) on both microsatellite and mtDNA sequence data. The Φ_{ST} analyses were performed using a matrix of Tamura & Nei (1993) distances. Observed heterozygosity at the microsatellite locus Ttho-6 was less than the expected heterozygosity in the majority of samples (see *Genetic variability*), likely caused by one (or more) null-allele(s), and was subsequently excluded from further analyses. In addition, three individuals from the Ty02 collection with missing data at microsatellite locus *Tth8* were excluded from the AMOVA analyses. ARLEQUIN was also used to calculate, θ, τ and Fu's F_s for the mtDNA data set. The θ-estimates (θ_0 and θ_1) are the product of $2\mu N_0$ and $2\mu N_1$ (where μ is equal to the mutation rate and *N* is the effective population size at times 0 and 1). Tau (τ) is a relative measure of time since population expansion, but can also be used to estimate the actual time (*T*) since a population expansion by $T = \tau/2\mu$ (where μ is equal to the mutation rate, Gaggiotti & Excoffier 2000). Fu's F_s tests whether mutations are neutral or under influence of selection, and

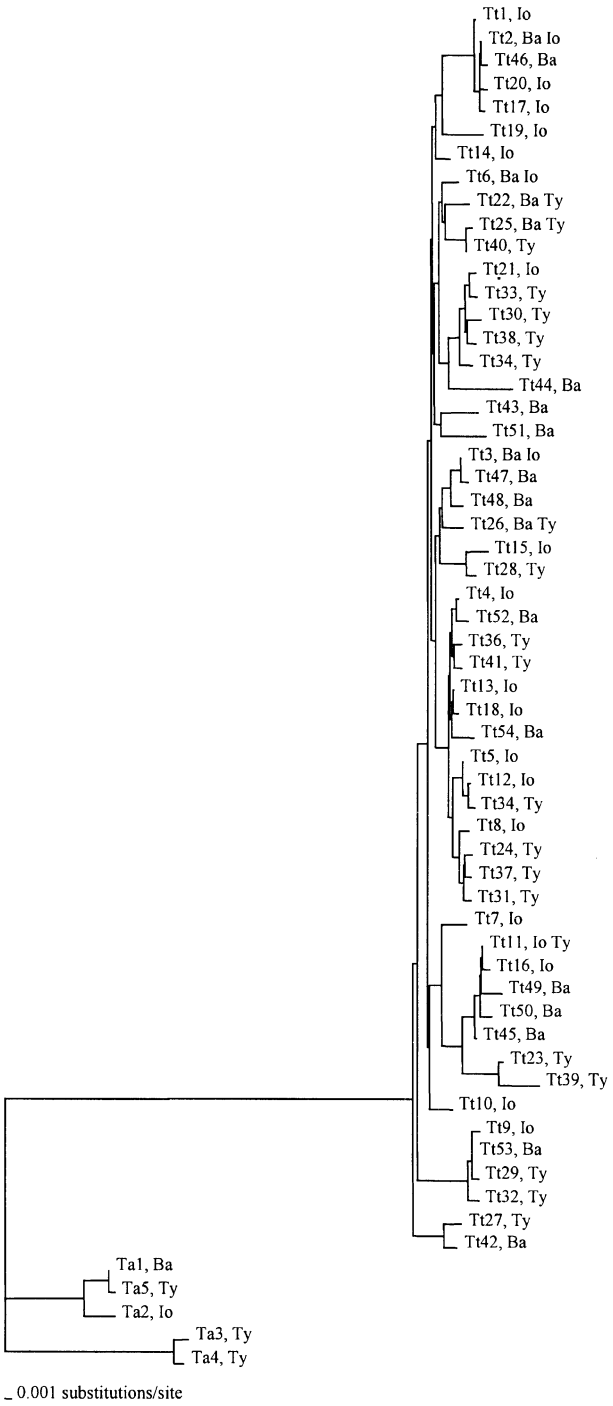

Fig. 2 Neighbour-joining tree of bluefin tuna mitochondrial control region sequences (868 bp) based on the Tamura & Nei (1993) distances. Ba, Balearic Sea; Ty, Tyrrhenian Sea; Io, Ionian Sea; Tt, bluefin tuna haplotypes; Ta, albacore-like haplotypes. See Electronic Appendix for information about specific haplotypes.

significantly negative values are also indicative of population expansion (Fu 1997). ARLEQUIN was used to estimate both Harpending's raggedness index (*Hri*; Harpending 1994) and mismatch distributions (*SDD*), which both test

whether the sequence data deviates significantly from the expectations of a population expansion model.

The program MIGRATE (Beerli & Felsenstein 2001; Beerli 2002) was used to estimate theta (Θ), which is equal to $4N_e\mu$ where N_e is the long-term (inbreeding) effective population size and μ is the mutation rate for the microsatellite data set. MIGRATE uses a Markov chain Monte Carlo-based maximum-likelihood (MCMC) approach to estimate Θ based on an expansion of the coalescence model (Kingman 1982a,b). Analyses involving microsatellite loci were performed under the 'allele model' as not all loci conformed to the strict expectations of the stepwise mutation model (SMM, alleles not conforming were only observed at low frequencies). Each MCMC run for the microsatellite loci consisted of 10 short chains (sampling 10 000 trees) and three long chains (sampling 100 000 trees) with a burn-in period of 10 000 trees. Three individuals from the Ty sample were excluded because of missing data at the *Tth8* locus. All runs were repeated five times to verify consistency of results and the data presented are the average of the five runs. When inspecting how the effective population size is estimated ($N_e = \Theta/4\mu$), it is evident that the mutation rate has a large influence on the estimate. The reported mutation rates (μ) for microsatellites range from 10^{-5} to 10^{-2} per locus and generation (Weber & Wong 1993); consequently, it is obvious that any estimate of effective population size is prone to very large variation (orders of magnitude) depending on which mutation rate is used. For this reason, we made no attempt to estimate the actual long-term inbreeding effective population size, but used the Θ-estimates as relative effective population sizes to compare between collections.

Results

Genetic variability

The number of alleles per microsatellite locus within samples varied from two at loci *Tth10* and *Tth21*, to 20 at locus *Ttho-6* (Table 1). Allele richness per locus and sample varied from 1.99 at locus *Tth10* in Ty98 to 10.18 at locus *Ttho-6* in Io99 (Table 1). Average observed heterozygosities varied from 0.222 in Io98 at locus *Ttho-1* to 0.929 in Ty98 at locus *Ttho-7* (Table 1), and average expected heterozygosities varied from 0.321 in Ty99 at locus *Tth10* to 0.935 in Io99 at locus *Ttho-6* (Table 1). The genotypic distribution at locus *Tth10* deviated significantly from Hardy–Weinberg expectations (heterozygosity excess) in Ba98 (Table 1). The genotypic distribution at locus *Ttho-6* deviated significantly from Hardy–Weinberg expectations (homozygosity excess) in the Ba98, Ba99, Ty99 and Io99 samples (Table 1). The MICRO-CHECKER analyses also showed that locus *Tth06* might be affected by null-alleles in Ty98, Ty99, Ty02, Ba98, Ba99 and Io99. In addition, the analysis suggested that locus *Tth8* in Ba99 might be affected by null-alleles. However, we could not find any indications that stuttering or large allele dropout affected our results. As locus *Tth06* probably was affected by null-alleles it was not included in further analyses. Since only the Ba99 sample showed indications of null alleles at locus *Tth8* (results from MICRO-CHECKER), and the locus *Tth10* only showed heterozygosity excess in the Ba98 sample, these loci were included in all analyses. To further assess if the microsatellite data were affected by mistyping of microsatellite alleles, we tested whether there was any correlation between global single locus F_{ST}s and number of alleles per locus, F_{IS} or H_T (Knutsen *et al*. 2003). None of these statistics was significantly correlated with the global single locus F_{ST}s (data not shown, $P = 0.159$, 0.193 and 0.167, respectively).

A total of 137 variable sites, including four indels, constituting 59 haplotypes was detected among the mtDNA control region sequences (868 bp) for the 74 bluefin tuna (Fig. 2 and Electronic Appendix). Five of 74 bluefin tuna (6.8%) had control region sequences that grouped closer with albacore than with bluefin tuna. Introgression of the albacore mitochondrial genome into bluefin tuna was reported by Chow & Kishino (1995). The five albacore-like sequences were not included in further analyses of mtDNA. However, because no private or rare microsatellite alleles were found in the five individuals with albacore-like haplotypes, these animals were consequently included in the microsatellite analyses. There were 82 variable sites (no indels) in the remaining 69 sequences, representing 54 haplotypes (Fig. 2 and Electronic Appendix). Haplotype diversity ranged from 0.984 to 0.996, and nucleotide diversity ranged from 0.011 to 0.020 (Table 2). A neighbour-joining tree based on the Tamura & Nei (1993) distance was drawn to visualize the relationship among different bluefin tuna haplotypes including the albacore-like haplotypes (Fig. 2).

Population differentiation and temporal stability

For the microsatellite data set, the sample sizes of the two Ionian Sea collections (total $n = 25$) were smaller than those from the Balearic and Tyrrhenian Seas (total $n = 134$ and $n = 121$, respectively). To avoid losing statistical power, the Ionian samples were excluded from the initial AMOVA. The comparison between the two collections from the western basin of the Mediterranean indicated no significant variation among collections from the same area in different years ($F_{SC} = -0.0013$, $P = 0.735$), and no significant spatial differentiation among the collections from the Balearic and Tyrrhenian seas ($F_{CT} = 0.0013$, $P = 0.196$; Table 3). A second AMOVA, which included the smaller Ionian sample, revealed no significant temporal variation within collections ($F_{SC} = -0.0020$, $P = 0.883$; Table 3), but demonstrated a significant amount of variation among collections ($F_{CT} = 0.0032$, $P = 0.019$; Table 3).

Table 2 MtDNA sequence variability in the control region, for bluefin tuna from the Mediterranean Sea: number of individuals (*n*), number of haplotypes (*nh*), haplotype diversity (*h*), nucleotide diversity (π), tau (τ), theta at time 0 and 1 (θ_0 and θ_1), Fu's F_s and Harpending's Raggedness index (*Hri*), and sum of squared differences from mismatch analyses (SSD)

Collection	*n*	*nh*	*h*	π	τ	θ_0	θ_1	Fu's F_s	*Hri*	SSD
Ba	24	19	0.982	0.013	12.193	0.002	92.734	−4.886*	0.0078	0.0029
Io	23	21	0.984	0.011	10.782	0.004	101.289	−10.196***	0.0132	0.0063
Ty	22	21	0.996	0.020	9.039	4.177	77.773	−13.189***	0.0084	0.0066
Total	69	54	0.991	0.015	11.708	0.007	81.133	−24.477***	0.0035	0.0009

Ba, Balearic; Ty, Tyrrhenian; Io, Ionian.
*, ***Significant at $P < 0.05$ and $P < 0.001$, respectively.

Table 3 AMOVA table of temporal and spatial genetic variation of bluefin tuna from three Mediterranean Sea locations, AMOVA I includes the Balearic and Tyrrhenian temporal replicates. The Ionian Sea temporal replicates are included in AMOVA II

	AMOVA I				AMOVA II			
Source of variation	% Of variation	Fixation indexes	*F*-statistics	*P*	% Of variation	Fixation indexes	*F*-statistics	*P*
Among groups	0.13	0.0013	F_{CT}	0.196	0.32	0.0032	F_{CT}	0.019
Among samples within groups	−0.13	−0.0013	F_{SC}	0.735	−0.19	−0.0020	F_{SC}	0.833
Within populations	100.00	−0.0000	F_{ST}	0.570	99.87	0.0013	F_{ST}	0.349

As no temporal variation was revealed by the AMOVA analyses, the temporal replicate collections from each location were pooled to increase statistical power. Pairwise multilocus F_{ST}s between the pooled collections were estimated to be 0.0007 between the Balearic and the Tyrrhenian Seas, 0.0046 between the Balearic and the Ionian Seas and 0.0087 between the Ionian and Tyrrhenian Seas, with only the latter being significantly different from zero ($P = 0.226$, $P = 0.103$ and $P = 0.015$, respectively). The global multilocus F_{ST} across the pooled collections was estimated to be 0.0023 ($P = 0.038$). Single-locus F_{ST}s were significant for locus *Tth10* between the Balearic and Tyrrhenian collections and for locus *Tth10* and *Tth04* between the Ionian and Tyrrhenian collections (data not shown). However, no single locus F_{ST} remained significant after sequential Bonferroni correction (initial $\alpha = 0.05/8 = 0.00625$).

An AMOVA using the Tamura & Nei (1993) distance performed on the mtDNA sequence data set revealed significant heterogeneity among the three collection locations ($F_{ST} = 0.0239$, $P = 0.0314$). Pair-wise Φ_{ST}s between the collections were estimated to be 0.0085 between the Balearic and the Ionian Seas, 0.0270 between the Balearic and the Tyrrhenian Seas, and 0.0366 between the Ionian and Tyrrhenian Seas ($P = 0.250$, $P = 0.053$ and $P = 0.030$, respectively). As with the microsatellite loci, only the Φ_{ST} between the Ionian and Tyrrhenian collections was found to be significantly different from zero. The global Φ_{ST} over all variable sites was estimated to be 0.0233 ($P = 0.000$).

To further test for evidence of population differentiation at the mtDNA locus, we calculated the nearest-neighbour statistic (S_{nn}) on the control region sequences. The test revealed a significant association between mtDNA control region sequence similarity and geographical location ($S_{nn} = 0.477$, $P = 0.007$).

Population expansion and effective population size

The ARLEQUIN analyses of mtDNA showed large differences in θ_0 and θ_1 within all collections, suggesting rapid population expansion (Table 2). All Fu's F_s-values were negative and significantly different from zero (Table 2). Mismatch distributions did not differ significantly from the distributions expected under population expansion in any of the collections (Table 2). The Harpending's raggedness index ranged from 0.0078 to 0.0132 (in the Ba and Io collections, respectively) and was estimated to be 0.0035 for the pooled sequences (Table 2). The τ-values (Table 2) were similar among collections and all had overlapping 95% C.I. (data not shown), indicating that the population expansions date back to roughly the same time period. The τ-value for the 69 pooled sequences was estimated to be 11.71 (95% C.I. 8.48–13.77). Reported mutation rates for the control region are variable, but we assumed an average mutation rate of 3.6×10^{-8} mutations per site and year, as this rate has been reported for the mtDNA control region in teleosts (Donaldson & Wilson

1999). Following the equation $T = \tau/2\mu$, the time since population expansion was estimated to be 187 156 years before present (95% C.I. 220 081–135 532).

The average Θ-values (from five runs) estimated with the MIGRATE software for the microsatellite data sets (each collection was represented by the pooled temporal replicates) were 0.748 (SE = 0.010) in the Balearic, 1.454 (SE = 0.166) in the Ionian and 0.777 (SE = 0.021) in the Tyrrhenian collection. The Θ-estimate for the Ionian collection differed significantly from both the Balearic and Tyrrhenian collections (Mann–Whitney Test, $P = 0.012$ for both tests), while no difference was found between the Balearic and Tyrrhenian collections (Mann–Whitney Test, $P = 0.296$).

Discussion

There has been considerable debate regarding the stock structure of North Atlantic bluefin tuna, and effective management of this species has been hindered by the lack of biological information needed to delineate appropriate management units (Restrepo *et al*. 2003). For several years scientists and managers have focused on determining whether bluefin tuna from the eastern Atlantic (including the Mediterranean Sea) and western Atlantic Ocean represent different (genetic) stocks. Our results suggest that the population structure of bluefin tuna might be even more complex than previously thought. Significant genetic heterogeneity among collections of YOY bluefin tuna from three locations in the Mediterranean Sea was found at both nuclear microsatellite loci and the mtDNA control region. For both classes of molecular markers, significant F_{ST} and Φ_{ST} estimates were found between collections from the Ionian and the Tyrrhenian Seas. In addition, the nearest–neighbour statistic (S_{nn}) indicated the presence of population structure as a significant association between mtDNA control region sequence similarity and geographical location was observed.

The observed heterogeneity among collections of YOY bluefin tuna from the Mediterranean Sea could be the result of sampling error, or alternatively biological/physical processes that promote reproductive isolation. For the microsatellite DNA analyses, the collection from the Ionian Sea comprised only 25 YOY bluefin tuna, while more than 120 individuals were screened from collections from the Balearic and Tyrrhenian seas. We recognize that small sample sizes limit the statistical power of an AMOVA, but it would most likely result in a failure to detect population structure (Type I error) rather than falsely indicating the presence of population structure. Furthermore, the results of the mtDNA analyses (AMOVA and S_{nn}), which were based on a similar number of individuals across the collections, corroborated the findings of the microsatellite analyses, reinforcing the observation of the genetic heterogeneity. Since the observed F_{ST} values were small, the possibility that the observed alleles could be influenced by miss-scoring was considered. However, approximately 20% of all individuals were run again to check for consistency in allele scoring. In addition, the MICRO-CHECKER analyses did not indicate null-alleles in any of the microsatellite loci included in our analyses, except for locus *Tth8* in the Ba99 sample, neither were there any indications of stuttering or large allele dropout. The global single locus F_{ST} values were not significantly correlated with either the number of alleles per locus, F_{IS}, or H_T, and thus it is unlikely that miss-scoring of alleles could explain the observed genetic heterogeneity. Finally, the smallest data sets, the Ionian collections, did not show any indications of reduced genetic variability due to small sample size (cf. *Rs* in Table 1) and thus the collections are probably representative of the population of origin.

It is also possible that the observed heterogeneity resulted from our use of YOY collections of bluefin tuna, which may represent the progeny of a small number of breeding adults rather than entire population (Allendorf & Phelps 1981). To test for such a bias, we screened genetic variability in at least two temporal replicates per location. No significant genetic differences were noted among collections taken in the same location during different years and no homozygosity excess (indicative of family effects) was observed, which is consistent with the assumption that the samples were not the progeny of a few individuals or affected by nonrepresentative sampling.

Previous genetic work on the Atlantic-wide population structure of bluefin tuna is not equivocal, as some indicate the presence of population structure while other studies do not (Edmunds & Sammons 1973; Thompson & Contin 1979; Broughton & Gold 1997; Alvarado Bremer *et al*. 1999; Takagi *et al*. 1999; Ely *et al*. 2002; Pujolar *et al*. 2003). Neither has there been a consensus about the population structure of bluefin tuna within the Mediterranean Sea; some studies report significant genetic heterogeneity while others do not (Broughton & Gold 1997; Alvarado Bremer *et al*. 1999; Pujolar *et al*. 2003; Viñas *et al*. 2003).

The discrepancy between our results and those from some earlier studies may be explained by the choice of markers and the age of sampled individuals. The large number of alleles and highly polymorphic nature of microsatellites has made them useful for the study of population genetic structure in marine fishes and microsatellites have been successfully employed to detect population structure in species where other markers have failed (Ruzzante *et al*. 1998; Shaw *et al*. 1999). Microsatellites and mtDNA control region sequences are also the only genetic markers to date that have successfully detected significant heterogeneity in bluefin tuna. Since no studies employing allozymes have found indications of population structure (but see Pujolar *et al*. 2003), it might be that allozyme variation is too low for allozymes to be suitable genetic markers for population

genetic studies of bluefin tuna. The significant genetic heterogeneity observed in the present study might result from the fact that we focused our collection efforts on YOY bluefin tuna. Previous genetic analyses of bluefin tuna population structure have been based on collections of subadults or adults (but see Ely *et al*. 2002 who included one temporal replicate from one location that was composed entirely of YOY bluefin tuna). Adult bluefin tuna, similar to other large pelagic fishes, have the potential to migrate over extensive distances (e.g. transatlantic movements; Mather *et al*. 1995; Block *et al*. 2001) and collections in a location may be composed of individuals originating from more than one spawning area. This could prevent the detection of genetic population structure. By restricting collections to YOY bluefin tuna, it is likely that our samples reflected the genetic composition of the spawning population better than if we had used older animals with greater migratory capacity (Graves *et al*. 1996). The observation of genetic heterogeneity among YOY bluefin tuna in the Mediterranean Sea suggests the existence of genetically independent population(s) of bluefin tuna in the eastern Mediterranean Sea. Bluefin tuna in spawning condition were recently reported in the eastern Mediterranean Sea (Karakulak *et al*. 2004) and were noted as early as 350 BC by Aristotle who claimed that bluefin tuna spawned along the coast of the Bosporus, which connects the eastern Mediterranean Sea and the Black Sea (Maggio 2000).

We did not observe significant genetic differentiation between YOY bluefin tuna from the Balearic Sea and the Tyrrhenian Sea, the two known spawning areas in the western Mediterranean Sea (Dicenta 1977; Garcia *et al*. 2001). This may result from significant levels of gene flow and/or physical mixing of individuals from these areas. Similar factors could be responsible for the lack of differentiation between the Balearic and the Ionian seas.

Both microsatellite and mtDNA analyses revealed the largest genetic differences between the two most proximate collections, the Ionian Sea and the Tyrrhenian Sea. This observation may be the result of oceanographic conditions within Mediterranean Sea that limit mixing of bluefin tuna eggs and larvae. The surface currents in the western Mediterranean basin are dominated by Atlantic water entering the Mediterranean Sea via the Strait of Gibraltar while cold, high-salinity water flows out of the Mediterranean Sea via the deeper areas of the Strait of Gibraltar (Millot 1999). The surface current continues on an eastern track until it reaches the Strait of Sicily, where it is divided into a stronger northern current bringing the water mass north of Sicily into the Tyrrhenian Sea, and a weaker south-east current that enters the eastern basin of the Mediterranean Sea (Millot 1999; Pinardi & Masetti 2000; cf. Fig. 1). The current entering the Tyrrhenian Sea continues north along the Italian coast and then follows the coast in a westward direction until it reaches the Balearic Islands (Millot 1999). This circulation could transport bluefin tuna eggs and larvae from the Tyrrhenian Sea to the Balearic Sea and promote physical mixing of individuals from the two spawning areas. Thus, the circulation pattern may be reflected in the lack of significant genetic differences between collections of bluefin tuna from the two locations. Similarly, the circulation pattern could be responsible for transporting proportionally more bluefin tuna larvae from the Balearic Sea to the Ionian Sea than from the Tyrrhenian Sea to the Ionian Sea. Oceanic currents may limit or hamper passive transport, and to some extent active movement, of bluefin tuna eggs and larvae between the western and eastern basins of the Mediterranean Sea. Rodríguez-Roda (1964) described two kinds of reproductive migrations of bluefin tuna through the Strait of Gibraltar. The first occurring through April to June, with bluefin tuna migrating into the Mediterranean Sea from the Atlantic Ocean, and the second in the opposite direction occurring in July and August comprising spent animals. We suggest that the observed heterogeneity of bluefin tuna within the Mediterranean Sea may be maintained by oceanic currents and spawning site fidelity.

The proportion of bluefin tuna found to have mtDNA control region sequences resembling albacore is interesting (cf. Fig. 2 and the Electronic Appendix), as mitochondrial introgression among tuna species has previously been reported (Chow & Kishino 1995). Viñas *et al*. (2003) showed that approximately 5% of the bluefin tuna in the Mediterranean Sea had mtDNA haplotypes similar to albacore, a proportion that corresponds well with our observation of five out of 74 (6.8%).

The τ-values for mtDNA were all in the same range among collections, indicating that all three collections likely originated from one colonization event between 220 000 and 135 000 years before present. The colonization was followed by rapid population expansion as seen in the large differences between θ_0 and θ_1 and the significantly negative Fu's F_s in all three collections. In addition, all Harpending's raggedness indices and mismatch distributions were non-significant, which should be expected when populations have undergone population expansion. A controversial theory, proposed by Arkhipov *et al*. (1995) and, later, Johnson (2002), suggests that during this period (144 000–137 000 years before present) the Mediterranean Sea was covered by a massive freshwater layer caused by extensive glacial runoff. Such a layer would have resulted in extensive changes in biotic and abiotic conditions, most probably rendering the Mediterranean Sea an unsuitable habitat for bluefin tuna. Thus, the bluefin tuna found in the Mediterranean Sea today probably colonized the basin some time after the end of the freshwater influx.

We employed MIGRATE to compare the long-term (inbreeding) effective population sizes at the three collection locations. The Θ-estimates from MIGRATE are directly related to

effective population size (N_e), but as the reported mutation rates for microsatellites vary greatly we refrained from using the Θ-estimates to assess actual effective population sizes. We assume, however, that the mutation rates for the microsatellites used in this study are the same in all studied populations, and thus it is valid to use the Θ-estimates for assessment of relative effective population size. The Θ-estimate for the Ionian collection was significantly larger than estimates for the Balearic and Tyrrhenian collections, suggesting that the long-term effective population size of bluefin tuna in the eastern basin of the Mediterranean Sea is larger than in the western basin of the Mediterranean Sea. The differences in Θ-estimates between the collections in the western and those in the eastern basins of the Mediterranean Sea further strengthen the suggestion of a genetically independent population of bluefin tuna in the eastern basin. Furthermore, the Θ-estimates in the Balearic and Tyrrhenian collections were not significantly different. This observation corroborates the lack of genetic differences between the Balearic and Tyrrhenian collections in the AMOVA analyses. Moreover, it is consistent with the expectation if these individuals were drawn from the same population.

Conclusions

The absence of temporal and spatial genetic differentiation as well as the similar Θ-estimates among bluefin tuna from the two proposed spawning areas in the western basin of the Mediterranean Sea implies a single population of bluefin tuna in this area. The genetic heterogeneity observed when including the bluefin tuna collection from the eastern basin of the Mediterranean Sea, combined with the recent finding of mature bluefin tuna in this area (Karakulak *et al*. 2004), suggests the potential for a genetically independent stock(s) of bluefin tuna the eastern basin of the Mediterranean Sea. Future studies of bluefin tuna population genetics in the Mediterranean Sea should incorporate more samples from the eastern basin of the Mediterranean Sea. If future studies verify the existence of one or more genetically independent bluefin tuna stocks in the eastern basin of the Mediterranean Sea, this should be taken into consideration when making decisions concerning the management and conservation of the species.

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2336/MEC2336sm.htm

Appendix S1. Variable sites matrix, distribution of mitochondrial control region haplotypes (Hap.) in bluefin tuna (Tt) and albacore like haplotypes (Ta) from collections from the Balearic (Ba), Ionian (Io) and Tyrrhenian (Ty) seas. GenBank accession numbers (GenBank). Variable sites within non-introgressed (Tt) bluefin tuna are in bold

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This paper is one in a series of ongoing studies of genetic population structure of large pelagic fishes conducted at the Department of Fisheries Sciences, Virginia Institute of Marine Sciences (VIMS), and at the Center for Biosystematics and Biodiversity, Texas A & M University (TAMU). The topic represents one of the major interests shared by the authors, namely population genetics, phylogeography and conservation genetics of marine and freshwater fishes. More information about the population genetics research conducted at VIMS and TAMU can be found at http://www.vims.edu/fish/fishgenetics/default.htm and http://wfscnet.tamu.edu/fl/fBBC.htm.
