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Evolutionary constraints limiting the variation of Expressed Sequence Tag-linked microsatellite loci, prevent the detection of local adaptation in Mediterranean Bluefin tuna

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ABSTRACT

The Atlantic Bluefin tuna (BFT, *Thunnus thynnus*), one of the largest top-predator fish inhabiting the pelagic ecosystems of the North Atlantic Ocean and Mediterranean Sea, has been extensively overexploited in recent decades. However, in the Mediterranean Sea, the mixing rates between the eastern, central and western basins have not yet been fully and finally resolved. To date electronic tagging, otolith and genetic markers cannot still disentangle the pattern of tuna movements and population structuring in the basin, essential background for a proper management of BFT fisheries. Here, we used Expressed Sequence Tag-linked (EST-linked) microsatellites to explore population dynamics and adaptive evolution of Mediterranean *T. thynnus*. For this purpose, 16 EST-linked microsatellites were genotyped in 177 tuna individuals from the Mediterranean Sea and several methods were used to explore population genetic structuring and estimate/detect signals of local adaptation. Bayesian clustering results indicated the presence of a single cluster, corroborated also by the Correspondence Analysis and pairwise F_{STs}. Similarly, the two methods used for the detection of outlier loci (LOSITAN and BayeScan), did not reveal any pattern suggesting the presence of selective pressure on the EST Simple Sequence Repeat (SSR) used. Our results suggest that the low level of polymorphism detected in this study could be ascribed to the presence of relatively conserved regions flanking these microsatellites. These genomic regions are probably not involved in physiological responses to local adaptation.

1. Introduction

The Atlantic Bluefin Tuna (BFT, *Thunnus thynnus*) is a large top-predator fish exploiting the pelagic ecosystems of the North Atlantic Ocean and Mediterranean Sea. While artisanal and low pressure fisheries for BFT have existed in the Mediterranean Sea for centuries (Addis et al., 2012), in recent decades the BFT fishing mortality rates have seriously increased (International Commission for the Conservation of Atlantic Tuna, 2014) leading to a continuous decline of the spawning stock biomass (Fromentin, 2009; MacKenzie et al., 2009) and to the risk of Western and Eastern stock collapse (MacKenzie et al., 2009). Definitely life-history traits make BFT vulnerable under continued excessive fishing pressure. BFT is indeed characterised by long life spans, large body size, late sexual maturity (around 4 years in the Mediterranean but up to 8 years for the Western Atlantic), geographically restricted spawning sites and relatively short spawning periods of 1 or 2 months (Fromentin and Fonteneau, 2001; Fromentin and Powers, 2005; Rooker et al., 2007; Fromentin, 2009; Juan-Jordá et al., 2011; Druon et al., 2016). The species has been managed by the International Commission for the Conservation of Atlantic Tuna (ICCAT) as two stocks and two populations, the western population, which spawn in the Gulf of Mexico, and the eastern population spawning in the Mediterranean. To date in the Mediterranean Sea the mixing rates between the eastern, central and western basins have not yet been resolved. Recent results (Cermeño et al., 2015) and prior works based on pop-up tracking systems (Block et al., 1998, 2001, 2005; Lutcavage et al., 1999; Rooker et al., 2008, 2014), support the existence of a western Mediterranean population distinct from an eastern Mediterranean population. Similarly genetic studies sug-

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gest there are at least three distinct populations throughout its geographical range (Carlsson et al., 2007; Boustany et al., 2008; Riccioni et al., 2010) that most likely represents Gulf of Mexico, western Mediterranean and eastern Mediterranean populations. Moreover within the Mediterranean there is evidence of complex population differentiation (Carlsson et al., 2007; Boustany et al., 2008; Cannas et al., 2012; Riccioni et al., 2010). Riccioni et al. (2013) have reported that BFT population structuring in the Mediterranean is related to oceanographic conditions, suggesting some kind of preferences for oceanographic areas that could explain the differentiation detected. Anyway a clear pattern of BFT structuring in the Mediterranean is still lacking.

Marine fish populations show typically low levels of genetic differentiation because they often have very large effective population sizes, which limit genetic drift, and relatively shallow histories (Hauser and Carvalho, 2008). Although some marine fishes often exhibit weak but significant population structuring even at small spatial scales (Bekkevold et al., 2005; Jorde and Ryman, 2007; Galarza et al., 2009; Zarraonaindia et al., 2012), a high number of neutral loci and large sample sizes are needed to resolve structure (Waples, 1998; Kalinowski, 2005). Moreover these neutral loci do not necessarily convey information on the extent or importance of adaptive variation (Larsen et al., 2007). Nonetheless the extent and dynamics of local adaptation is the key to understand the ecological and evolutionary processes that influence biodiversity, as well as to provide a spatially explicit framework for the conservation of genetic resources (Salmenkova, 2011; Funk et al., 2012; McMahon et al., 2014).

The Expressed Sequence Tags (ESTs) are a rich source of many Simple Sequence Repeats (SSRs) (Ellis and Burke, 2007) and EST-linked SSRs (EST SSRs) display several valuable features to disentangle the extent and dynamics of local adaptation in marine fish populations (Naish and Hard, 2008). These features include, as neutral microsatellites, their abundance in the genome, high levels of polymorphism, locus-specific codominance, higher clarity of scoring related to a more conservation level of DNA flanking ESTs and, most relevantly, the potential to be loci under selection because of their linking to expressed genes (Ellis and Burke, 2007). It is likely that ecologically important traits have been shaped by natural selection, which means that examining patterns of molecular evolution in EST-linked microsatellites could provide a way of screening numerous genetic loci for signatures of adaptive evolution.

In this study we genotyped 16 species-specific EST-linked microsatellite loci (Molecular Ecology Resources Primer Development Consortium et al., 2010) in four samples previously analysed by using neutral microsatellite loci (Riccioni et al., 2010, 2013), to test population genetic differentiation of *T. thynnus* in the Mediterranean possibly linked to adaptation and to obtain a more clear picture of BFT population dynamics in the area.

2. Materials and methods

Tissue samples of the Atlantic Bluefin tuna *Thunnus thynnus* used in this study were collected from Mediterranean individuals caught during scientific research programs under the permission of the Italian Ministry of Agricultural and Forestry Policies (locations: Adriatic Sea, ADR) and by commercial long-liners and purse-seiners within the Total Allowed Catch quotas assigned by the ICCAT to National Governments (locations: Alboran Sea, ALB; Tyrrhenian Sea, STY; Cyprus coasts, CYP). The complete dataset included 4 population samples of BFT (N = 177, Fig. 1 and Supplementary data Table 1). DNA extraction and EST-linked microsatellite PCR amplification were performed according to Molecular Ecology Resources Primer Development Consortium et al. (2010). All the PCR included negative controls and PCR products were resolved on 1.5% agarose gels. The allele sizing was carried out on an ABI Prism 3100 automatic sequencer using fluorescent-labelled forward primers (Molecular Ecology Resources Primer Development Consortium et al., 2010), using the LIZ 500 as internal standard and the software GeneScan Analysis v. 2.02 (Applied Biosystems).

The software Microchecker (Van Oosterhout et al., 2004) was used to check microsatellite data for null alleles and scoring errors. The number of alleles, the expected (H_e) and observed (H_o) heterozygosity per locus and per sample, and the corresponding exact test for Hardy–Weinberg Equilibrium (HWE) were calculated by ARLEQUIN v.3.5 (Excoffier and Lischer, 2010; Excoffier et al., 2005) after 1,000,000 steps of Markov chains and 100,000 dememorization steps. ARLEQUIN v. 3.5 was also used to estimate pairwise F_{ST} (Weir and Cockerham, 1984) using 10,000 permutations to obtain the null distribution of F_{ST} under the hypothesis of panmixia. A global HWE test was performed by GENEPOP 4.0 (Raymond and Rousset, 1995) using 10,000 dememorization steps of Markov chains, 20 batches and 5,000 iterations per batch. The allelic richness for each locus and population were computed by using FSTAT 2.9.3 (Goudet, 2001) to compare samples of different sizes. The sequential Bonferroni's correction for multiple simultaneous tests (Rice, 1989) was applied where needed. The software STRUCTURE 2.3 (Pritchard et al., 2000; Falush et al., 2003; Hubisz et al., 2009) estimates Pr (X|K), the probability of the data given K genetic clusters of individuals (K = 1, 2...), by a Bayesian model-based algorithm under the HWE assumption. STRUCTURE 2.3 was run allowing the use of sampling location information and admixture model with correlated allele frequencies. Ten independent analyses were run each with a different value of K (1–10). Each run of analysis consisted in 1,000.000 Monte Carlo Markov Chains with a burnin period of 500,000. The most likely number of clusters was inferred using both the standard method, plotting ln Pr (X|K) vs K and using the Bayes' rule, Pritchard et al., 2000) and the ΔK statistic (Evanno et al., 2005) based on a rate of change in the log probability of the data. The results were averaged over multiple runs using the CLUMMP software (Jakobsson and Rosenberg, 2007) and displayed using the DISTRUCT program (Rosenberg, 2004). The Correspondence Analysis (CA; Greenacre, 1984; Jombart et al., 2009) was used to further investigate the spatial pattern of genetic variability among tuna samples. The CA was performed using the R package ADE4 1.4 (Dray and Dufour, 2007) and ADEGENET 2.7 (Jombart, 2008). The CA is an 'ordination in reduced space' method that optimizes the χ^2 distances among observations and therefore it can give a stronger weight to a population possessing a rare allele. As a consequence, to minimize analysis artefacts, alleles present in single copy in only one population were removed. Moreover two different methods were applied to evaluate the presence of F_{ST} outliers to test for selection signals and identify candidate loci. The FDIST2 approach of Beaumont and Nichols (1996) implemented in LOSITAN (Antao et al., 2008) was used by simulating 50,000 distributions with a 95% confidence interval and a false discovery rate of 0.1 for neutrally distributed markers with both the stepwise and infinite allele mutation model. These distributions are used to identify outlier loci that show excessive high or low F_{ST} compared to neutral expectations. BayeScan 2.1 (Foll, 2012) aims at identifying candidate loci under natural selection from genetic data, using differences in allele frequencies between populations. It uses the island model and the multinomial-Dirichlet model to describe several ecological scenarios and it implements a reversible-jump MCMC algorithm to estimate the posterior probability of each one of these models. For this analysis, following 20 pilot runs of 5,000 iterations and an additional burn-in of 50,000 iterations, we used 100,000 iterations (sample size of 5,000 and thinning interval of 20) to identify loci under selection from locus specific Bayes factors.