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## NOTE

# Potential Impact of Mediterranean Aquaculture on the Wild Predatory Bluefish

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## Abstract

Aquaculture impacts on wild populations of fish have been considered principally due to farm escapes. The Bluefish *Pomatomus saltatrix*, which exhibits two distinct genetic units in the Mediterranean Sea, is a voracious predator and is attracted to aquaculture cages to prey on farmed fish, particularly Gilthead Seabream *Sparus aurata* and European Sea Bass *Dicentrarchus labrax*. We compared the genetic diversity of adult Bluefish caught inside one aquaculture farm located in Spanish waters of the western Mediterranean Sea with reference individuals of East and West Mediterranean stocks from the open sea. Bluefish were genetically assigned to their putative origin using seven microsatellite loci and mitochondrial cytochrome oxidase subunit I as molecular markers. As expected, most of the individuals caught from inside the fish farm cages were assigned to the local genetic population. However, between 7.14% and 11.9% of individuals were assigned to the distant and different genetic unit inhabiting Turkish waters, the East Mediterranean stock. The genetic membership of those individuals revealed some degree of interbreeding between the East and West Mediterranean Bluefish stocks. All results suggest that aquaculture

acts as an attractor for Bluefish and could affect genetic diversity as well as phylogeography of this fish and maybe other similar species that aggregate around marine fish farms.

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The impacts of aquaculture on natural ecosystems are wide-ranging, from esthetic aspects to undesirable effects on wild surrounding populations (Black 2000; Fernandes et al. 2002; Diana 2009). One of the most studied effects on wild fish populations is the genetic interaction between farm escapees and wild conspecifics (e.g., Hindar et al. 1991; Youngson et al. 2001; Read and Fernandes 2003; Naylor et al. 2005). Variants from domestic fishes can be introduced into native genetic pools thus reducing local adaptation, as reported for different species (e.g., Youngson et al. 2001; Utter and Epifanio 2002). Farm escapees and deliberate releases of hatchery stocks also have the potential to alter population structuring and

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phylogeographic patterns of aquatic species. Examples are abundant and include salmonids (e.g., Machordom et al. 2000; Bernatchez 2001; Naylor et al. 2005), flatfishes (e.g., Danancher and Garcia-Vazquez 2011), shellfish (e.g., Gaffney 2006), and algae (e.g., Voisin et al. 2005). Interspecific impacts of aquaculture have also been reported. These include the reduction of local native populations by competition with exotic farmed species (Naylor et al. 2000; McGinnity et al. 2003), as well as increases in interspecific hybridization (Verspoor 1988; Youngson et al. 1993), sometimes due to alterations of behaviour in domestic variants (Hutchings and Fraser 2009; Castillo et al. 2010). In general, the study of the genetic impacts of aquaculture has focused on wild populations of the same species and/or closely related species that can hybridize with escapees.

Nevertheless, there are other heterospecific impacts that could also be derived from aquaculture. Hatcheries and aquaculture cages attract predators like bottlenose dolphins *Tursiops truncatus* (Díaz-López et al. 2005; Díaz-López and Bernal-Shirai 2007), cormorants (Díaz-López et al. 2008; Liordos and Goutner 2008; Akyol and Ertosluk 2010), and many wild fish species (Dempster et al. 2002; Valle et al. 2007). However, one aspect that has rarely been considered is how the attraction an aquaculture facility can potentially change the genetic pattern of an attracted species. If fish farms are a magnet for predators that repeatedly change their migratory routes, we might expect that the population structure and, on a longer term, the phylogeographic pattern of the attracted species can change. Those changes can alter the ecosystem equilibrium and may potentially result in its collapse (e.g., McCann 2007; Baum and Worm 2009), but the consequences of a phylogeographic change of predator species have not been explored yet.

We used Bluefish *Pomatomus saltatrix* as a case study to investigate the effects of aquaculture farms on attracted predators. Bluefish is a fast-growing piscivorous predator (e.g., Juanes and Conover 1994) that has been reported to stay around fish farms, break into sea cages, and feed on farmed fish (e.g., Gilthead Seabream *Sparus aurata*) in the western Mediterranean Sea near Alicante, Spain (Sanchez-Jerez et al. 2008; Arechavala-Lopez et al. 2015). Bluefish exhibit spatial population differentiation across the Mediterranean Sea (Pardiñas et al. 2010; Miralles et al. 2014b) and are good candidates for testing the effects of attraction at long-distance scales since individuals from different areas can be identified by their genetic pattern. Our hypothesis was that Bluefish from inside the farm are a mixture of genetic lineages coming from different geographical areas, and as a consequence, this might alter the phylogeographic pattern of the species.

## METHODS

*Study site and sampling.*—A total of 159 Bluefish were collected during 2004 and 2005 along the coast of the Mediterranean Basin. Bluefish is a highly migratory species composed of different stocks and populations along its cosmopolitan distribution. In the study site, two distinct genetic units called East and West Mediterranean were previously described (Pardiñas et al. 2010; Miralles et al. 2014b), and there is an indication of some connectivity between them (Miralles et al. 2014a).

Spearfishing was used to collect 42 Bluefish inside the Gilthead Seabream and European Sea Bass *Dicentrarchus labrax* marine farm cages located 3.7 km from shore in Guardamar (Alicante, Spanish Mediterranean coast; Figure 1). Created in

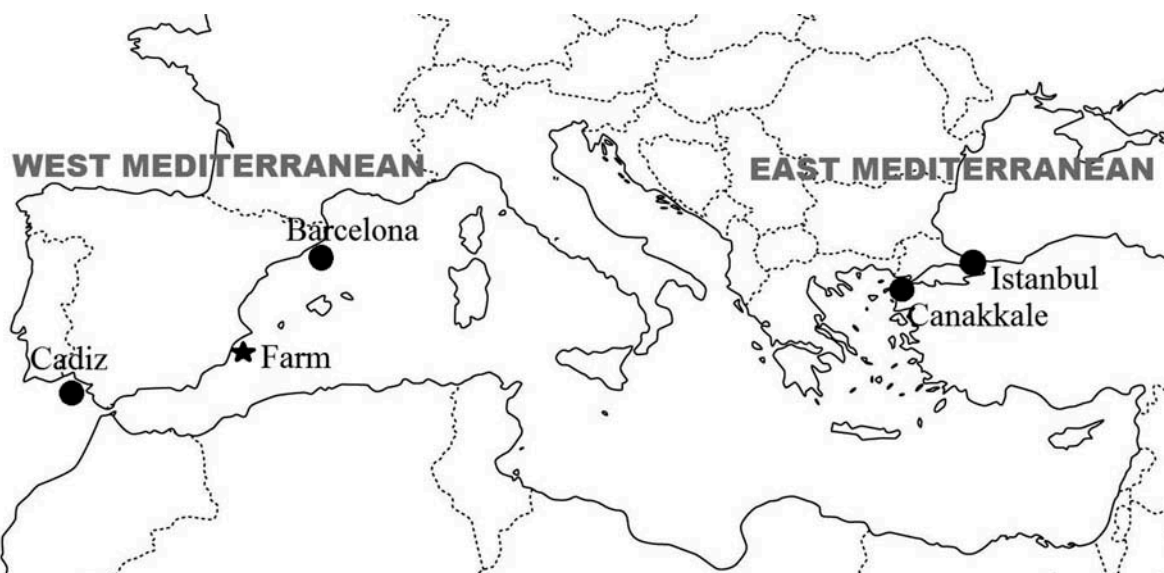


FIGURE 1. Bluefish sampling locations in the western and eastern Mediterranean Sea.

2001, this fish farm is one of the largest aquaculture farms in Spain and produces more than 3,500 metric tons of Gilthead Seabream and European Sea Bass per year. Moreover, in the Mediterranean Sea this farm is the one most affected by repeated and frequently reported intrusions of Bluefish inside the facilities (Sanchez-Jerez et al. 2008; Arechavala-Lopez et al. 2011, 2015). Samples of adult and mature Bluefish that had broken the netting and were inside sea cages of this western Mediterranean fish farm were fished on several randomly selected days throughout the sampling period.

Reference wild populations of Bluefish from the open sea were defined according to the two distinct genetic units described in the Mediterranean Basin (Pardiñas et al. 2010; Miralles et al. 2014a, 2014b). For the East Mediterranean genetic unit, the reference samples ( $n = 44$ ) came from two sites: Istanbul in the Sea of Marmara ( $n = 24$ ) and Canakkale in the Aegean Sea ( $n = 20$ ), both within Turkey and located at approximately 2,900 and 2,700 km, respectively, from the Guardamar aquaculture facilities. For the West Mediterranean Bluefish genetic unit, the samples ( $n = 72$ ) were collected near Barcelona in the western Mediterranean Sea ( $n = 7$ ) and in the Gulf of Cadiz in the Atlantic Ocean ( $n = 65$ ), both within Spain and at 500 and 900 km, respectively, from Guardamar.

*DNA extraction, amplification and sequencing.*—Small pieces of Bluefish muscle were dissected and preserved in absolute ethanol prior to laboratory analyses. The DNA was extracted following a Chelex-based protocol (Estoup et al. 1996). Seven hypervariable microsatellite loci were PCR-amplified following the conditions described by Dos Santos et al. (2008). Allele sizes were determined from PCR products using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) in the Unit of Genetic Analysis at the University of Oviedo, Spain. Microsatellite alleles were scored with the program GeneMapper version 4.0 (Applied Biosystems).

The mitochondrial cytochrome oxidase subunit I (COI) gene was amplified with the primers, conditions, and protocol described in Miralles et al. (2014a, 2014b). Sequencing was performed using the genetic analyzer at the University of Oviedo with a BigDye 3.1 Terminator system.

*Population genetic diversity and differentiation estimates.*—The program MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004) was used to check for null alleles, scoring errors, and allele drop-out. Microsatellite variation (number of alleles per locus, allelic richness, and observed and expected heterozygosities) was calculated with the programs GENETIX version 4.03 (Belkhir et al. 2001) and FSTAT version 2.9.3.2 (Goudet 2001). Exact tests for the departure from Hardy–Weinberg equilibrium were performed with GENEPOP version 1.2 (Raymond and Rousset 1995) using Bonferroni corrections.

Sequences of COI were edited with BioEdit Sequence Alignment Editor (Hall 1999) and aligned with ClustalW (Thompson et al. 1994). The program DNAsp version 5 (Librado and Rozas 2009) was employed to calculate the

number of haplotypes ( $N_h$ ), haplotype diversity ( $H_d$ ), and nucleotide diversity ( $\pi$ ). Pairwise  $F_{ST}$  values, indicators of genetic distance between populations, were calculated with Arlequin version 3.0 (Excoffier et al. 2005) (10,000 permutations, 100,000 steps in Markov chain).

*Population assignment based on genotypes.*—To examine assignment accuracy a pretest based on leave-one-out individual assignments was performed using the software ONCOR (Kalinowski et al. 2007), taking into account that 90% correct assignment is the threshold often used as an indication that the baseline stocks have been adequately delineated for assigning individuals from mixed fisheries. Furthermore, Bluefish assignment to a population of origin was carried out employing three different methodologies to get more robust conclusions based on consistent results. We used GeneClass2 (Piry et al. 2004) for Bayesian assignment with a 0.05 score threshold, and two methods were assayed: Rannala and Mountain (1997) and Baudouin and Lebrun (2001). Assignment results were checked by Monte Carlo resampling through the simulation algorithm of Paetkau et al. (2004) with 10,000 individual simulations and a type I error of 0.01. We used ONCOR (Kalinowski et al. 2007) for maximum-likelihood assignment tests. Individuals were assigned to a population based on the probability of that population containing the individual's genotype. Also, a classical mixed stock analysis (MSA) was conducted using the Statistical Program for Analyzing Mixtures (SPAM) version 3.7 (Alaska Department of Fish and Game 2003) with 10,000 iterations and 1,000 bootstraps.

To check the possibility of mixed ancestry of individuals from inside the farm or possible interbreeding between East and West Mediterranean Bluefish stocks we employed STRUCTURE 2.3.1 (Pritchard et al. 2000). Data sets were analyzed under the “Admixture model,” which assumes that individuals may have mixed ancestry. The parameter set consisted of a burn-in period of 100,000 steps followed by 1,000,000 Markov chain Monte Carlo (MCMC) iterations and five runs for  $K = 2$  (number of genetic units).

## RESULTS

### Population Genetic Diversity and Differentiation

Bluefish collected inside the Guardamar fish farm exhibited very high genetic diversity at both microsatellite and mitochondrial DNA, even higher than those from all the other sampling locations clustered together (Figure 2A) and significant in a Wilcoxon signed-rank test ( $W$ -value = 0,  $P \leq 0.05$ ). We found a total of 12 COI sequence haplotypes (accession numbers: JQ039400–JQ039406, JQ039425–JQ039429 in GenBank; [www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/)); half of these haplotypes were found only in specimens caught inside the farm. Bluefish inside the

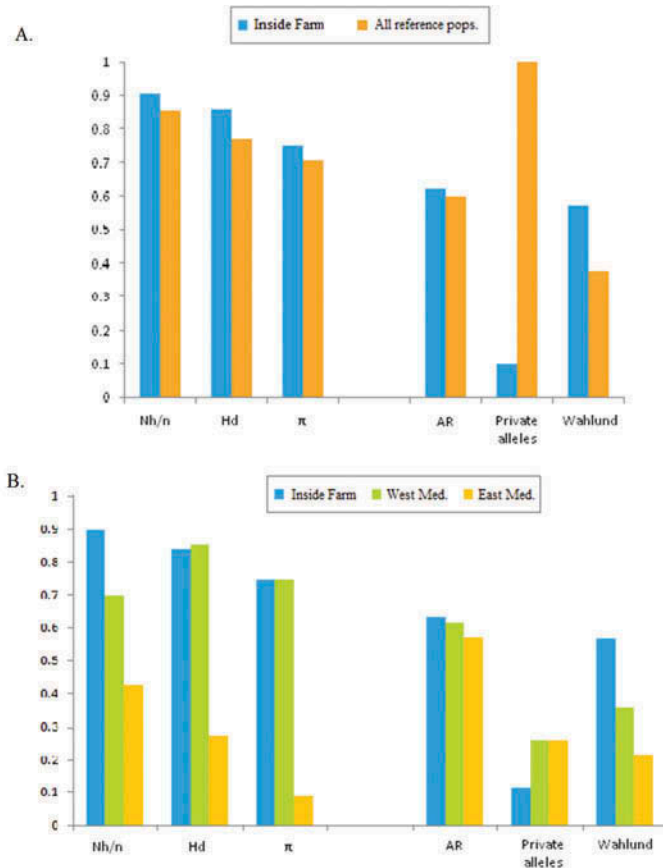


FIGURE 2. Genetic diversity parameters for Bluefish from inside the Spanish aquaculture farm compared (A) with all reference populations together and (B) with West and East Mediterranean reference populations separately: variation at mitochondrial DNA (left graphs) and microsatellite loci (right graphs). Nh: number of haplotypes;  $n$ : number of individuals; Hd: haplotype diversity;  $\pi$ : nucleotide diversity; AR: allelic richness; Wahlund: loci with significant Wahlund effect (excess of homozygotes, an indicator of population mixture). Values were transformed mathematically for easier visualization and comparison in the graph as:  $Nh/n (\times 2)$ ;  $\pi (\times 300)$ ; AR and private alleles ( $\div 20$ ); Wahlund ( $\times 10$ ).

farm had higher COI diversity (Figure 2A, left panel) than all the other Bluefish samples analyzed in this study (obtained from four distant locations). Bluefish inside the farm were also more diverse at microsatellite loci than all samples from outside the farm (Figure 2A, right panel), exhibiting higher allelic richness, more loci with possible Wahlund effects (excess of homozygotes, but not significant deviations from Hardy–Weinberg equilibrium after Bonferroni corrections except for one locus in one location), and fewer private alleles. A similar pattern was also visible when samples from the two reference genetic units were compared: East and West Mediterranean (Figure 2B.). All of these results suggest that the high diversity of Bluefish caught inside the farm was due to population mixture.

### Putative Population Assignment Based on Genotypes

Based on leave-one-out analyses, incorrect assignments were lower than 6%; thus, the reported Bluefish population assignments can be considered robust. The three population assignment methods showed that the majority of Bluefish caught inside the farm belonged to the local reference population, the West Mediterranean genetic unit (Figure 3). Surprisingly, a high percentage of individuals was assigned to Turkish populations (Figure 3; Table 1), varying from 7.14% to 11.9% depending on the methodology used. The three methods used in this study assigned the same three individuals (labeled A09, A21, and A35) to the Turkish population (Figure 3). Two more individuals were identified by at least one Bayesian method: individual A36 with the Rannala and Mountain (1997) method and individual A42 with the Baudouin and Lebrun (2001) method. Furthermore, employing a classic MSA approach, 10.8% of Bluefish caught inside the farm belonged to the East Mediterranean stock (95% CI, 0.0–26.6%).

Mixed ancestry was detected in the individuals of non-local origin that were caught inside the farm (Table 2). Proportions of East Mediterranean stock membership varied from 24.6% to 92.8% inside the farm suggesting interbreeding between the two lineages, while in the reference populations only 11.08% of mixed membership in Turkish and 14.05% in Spanish stocks were found.

### DISCUSSION

Because there is a continuous global increase in marine aquaculture the effects this may have on a wider ecosystem scale should be of concern. In this study, we examined the possible evolutionary changes that may occur in the surrounding accompanying species due to aquaculture, using a predator fish species, the Bluefish, as a case study. Using different assignment methodologies with different approaches and a mixed stock analysis, we obtained similar results: Bluefish caught inside the aquaculture farm belonged to different genetic units from the two subbasins of the Mediterranean Sea. They likely came from different regions of the Mediterranean Sea, including distant Turkish localities, and also the closer north-eastern Atlantic Ocean in Spain, thus making up a mixture of immigrants of different genetic stocks. Accordingly, our results showed that the Bluefish found inside the farm had higher genetic diversity than all the reference samples clustered together and formed a mixed population containing lineages from different genetic units previously described in the Mediterranean Basin (Pardiñas et al. 2010; Miralles et al. 2014b).

In a comparison of the three different methodologies (ML, Bayesian, and MSA), ONCOR assignments were stricter than the two GeneClass methods. The Rannala and Mountain (1997) method, frequently employed to

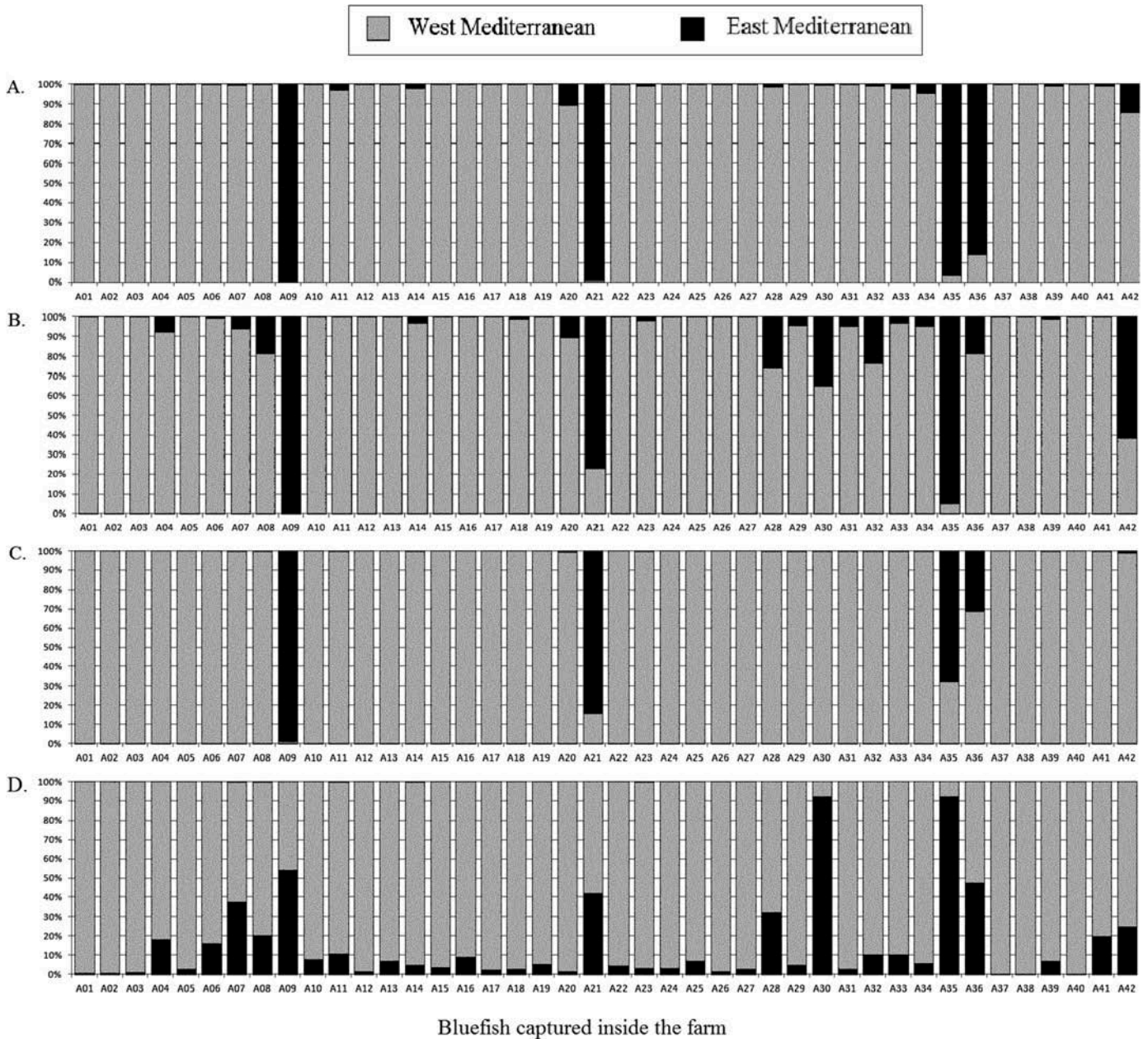


FIGURE 3. Assignment scores and membership estimations of Bluefish from inside the aquaculture farm. Each bar represents one individual fish from A1 to A42 belonging to the farm. Assignment to the local population (West Mediterranean) is in gray and assignment to Turkish population (East Mediterranean) is in black. (A) Rannala and Mountain (1997) method; (B) Baudouin and Lebrun (2001) method; (C) ONCOR (Kalinowski et al. 2007) method; (D) STRUCTURE software (Pritchard et al. 2000).

assign fish to farms by other investigators (Glover et al. 2008, 2009), yielded the highest assignment values to Turkish populations (Table 1; Figure 3A). The Baudouin and Lebrun (2001) method was intermediate. Finally, the MSA method did not identify the individuals but provided a CI for the results. Therefore we recommend combining all the methodologies for the best resolution and robust analysis. From this perspective, a minimum of 7% and a

maximum of 12% of farm-collected Bluefish individuals would be immigrants. Results also suggest that the observed differentiation of Bluefish between the western and eastern subbasins of the Mediterranean Sea (Pardiñas et al. 2010; Miralles et al. 2014b) are in a regressive process if the immigrants stay in the farms and reproduce, and then the two genetic units fuse together. This process has already been detected since those immigrants had

TABLE 1. Immigrant frequencies for different assignment scores based on each methodology: a summary comparing the three assignment methodologies based on four assignment thresholds (90, 80, 70, or 60% probability of belonging to a putative population). GC2–RM: GeneClass2, Rannala and Mountain (1997) method; GC2–BL: GeneClass2, Baudouin and Lebrun (2001) method; ONCOR: ONCOR (Kalinowski et al. 2007) method; GC2 + ONCOR: a combination of the three methodologies.

Assignment method	Assignment threshold			
	90%	80%	70%	60%
GC2–RM	0.07	0.10	0.10	0.10
GC2–BL	0.05	0.05	0.07	0.10
ONCOR	0.02	0.05	0.05	0.07
GC2 + ONCOR	0.02	0.10	0.10	0.12

TABLE 2. East and West Mediterranean lineage membership of Bluefish caught inside the fish farm. Individual membership to East (in bold text) and West Mediterranean genetic clusters were estimated with STRUCTURE software. The probability of belonging to the East Mediterranean cluster was obtained with three independent assignment methods: GC2–BL: GeneClass2, Baudouin and Lebrun (2001); GC2–RM: GeneClass2, Rannala and Mountain (1997); ONCOR: Oncon Software (Kalinowski et al. 2007). Membership and probability values are given in percent.

Individual immigrants	Lineage membership (%)			
	STRUCTURE	GC2–BL	GC2–RM	ONCOR
A09	<b>54.3</b> /45.7	66.2	97.4	98.4
A21	<b>42.6</b> /57.4		92.4	86.8
A28	<b>32.0</b> /68.0	58.3		
A30	<b>92.0</b> /8.0	47.1		
A35	<b>92.5</b> /7.5	63.0		
A36	<b>47.9</b> /52.1	63.7	57.4	
A42	<b>24.6</b> /75.4	73.5	95.0	

mixed ancestry. In other words, the two stocks seem to interbreed. The effects of this evolutionary process may depend on several factors such as the history and size of the populations as well as the time scale (Hauser and Carvalho 2008). However, the estimate of 7–12% immigrants inside the farm should be interpreted with caution given that the leave-one-out analysis showed a misassignment rate of almost 6% in the known-origin reference samples. Also, that unsampled Bluefish source populations might exist and only one aquaculture farm was studied should be taking into account.

This study provides a new perspective on aquaculture effects on the surrounding ecosystem. Aquaculture facilities might have an impact on the phylogeography of wild predators such as Bluefish that are not farmed but are attracted to fish farms. In conclusion, based on genetic assignments of Bluefish caught inside a Spanish Gilthead Seabream farm, we revealed that there is a mixture of East and West Mediterranean lineages inside the farm located in Guardamar in the western Mediterranean Sea. We suggest that marine farms modify the genetic variation of accompanying species at different levels, from local population diversity to phylogeographic scale, especially when

prolonged attraction occurs from a long distance, as it seems to happen in the Mediterranean Sea.

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