

TEÓFILO DE SALES MORIM

**INVASIVE GENETICS OF THE MUMMICHOG  
(*FUNDULUS HETEROCLITUS*): recent anthropogenic introduction in  
Iberia**



UNIVERSIDADE DO ALGARVE  
Faculdade de Ciências e Tecnologia  
2016/2017

TEÓFILO DE SALES MORIM

**INVASIVE GENETICS OF THE MUMMICHOG  
(*FUNDULUS HETEROCLITUS*): recent anthropogenic introduction in  
Iberia**

Mestrado em Biologia Marinha

Trabalho efetuado sob a orientação de:

Professora Doutora Rita Castilho

Doutora Regina Cunha



UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2016/2017

## **Declaração de autoria de trabalho**

INVASIVE GENETICS OF THE MUMMICHOG (*FUNDULUS HETEROCLITUS*):  
recent anthropogenic introduction in Iberia

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Teófilo de Sales Morim

(Teófilo de Sales Morim)

A Universidade do Algarve reserva para si o direito, em conformidade com o disposto no Código do Direito de Autor e dos Direitos Conexos, de arquivar, reproduzir e publicar a obra, independentemente do meio utilizado, bem como de a divulgar através de repositórios científicos e de admitir a sua cópia e distribuição para fins meramente educacionais ou de investigação e não comerciais, conquanto seja dado o devido crédito ao autor e editor respetivos.

## **Agradecimentos**

À Professora Doutora Rita Castilho, pela sua constante disponibilidade, imenso apoio e sábia orientação, que permitiram a construção deste trabalho.

À Doutora Regina Cunha, pela motivação e apoio, e em especial o seu incansável contributo para a otimização de um dos protocolos.

Ao Pedro Madeira, pelo acompanhamento constante ao longo do processamento das amostras.

Ao Doutor Jorge Palma do Centro de Ciências do Mar (CCMAR, Portugal), à Professora Mila Soriguer da Universidade de Cádiz em Espanha, ao Doutor David D. Duvernell da Universidade do Sul de Illinois - Edwardsville (EUA) e ao Doutor Enric Gisbert do Centro de Aquacultura do Instituto de Investigação e Tecnologia Agroalimentar (IRTA, Espanha), pelo seu papel crucial na recolha das amostras em, respetivamente, Faro, Cádiz, costa este da América do Norte e Delta do Ebro.

À equipa do laboratório de Biogeografia, Ecologia e Evolução do Centro de Ciências do Mar por manterem um excelente ambiente de trabalho, propício à partilha de conhecimentos e entreaajuda.

## Resumo

*Fundulus heteroclitus* é um pequeno teleósteo extremamente resistente, capaz de sobreviver a uma larga gama de temperaturas e salinidades. É uma espécie endêmica da costa Atlântica da América do Norte, onde pode ser encontrada quase continuamente desde a Terra Nova até à Flórida em zonas costeiras, estuários e sapais. Uma vez que apresenta capacidade de dispersão muito reduzida é uma espécie que raramente sai do ambiente que habita. Não só a biologia desta espécie já foi extensivamente estudada, como é também considerada uma espécie modelo para o estudo de variações clinais. Vários estudos focados na sua distribuição nativa demonstraram diferenças significativas a nível morfológico, genético e fisiológico ao longo da costa da Nova Jérnia (40-41° N), o que promoveu a divisão em duas subespécies: *F. heteroclitus macrolepidotus* com uma distribuição mais a norte e *F. heteroclitus heteroclitus* localizado a sul. *F. heteroclitus* foi encontrado pela primeira vez na Península Ibérica na costa sudoeste de Espanha na década de 70, tendo sido provavelmente introduzido entre 1970 e 1973. Desde então, estabeleceu-se com sucesso nesta região, podendo ainda ser encontrado na zona costeira da Ria Formosa em Portugal e no Delta do Ebro em Espanha. Neste estudo analisámos sequências obtidas a partir de um marcador mitocondrial, citocromo *b*, de modo a elucidar o padrão de expansão de *F. heteroclitus* na Península Ibérica. As sequências foram recolhidas a partir de indivíduos provenientes de três populações invasoras na Península Ibérica (Faro, Cádiz e Delta do Ebro) e de 13 outras localizações ao longo da sua distribuição nativa. A análise das sequências compostas por 700 pares de bases revelou a presença de um único haplótipo comum a todos os indivíduos invasores provenientes da Ibéria. A extrema ausência de diferenciação e estrutura genética das populações Ibéricas é consistente com uma invasão recente desde a qual apenas se passaram cerca de 40 anos, e indica que estas populações invasores sofreram um forte efeito fundador: a primeira população resultou de um pequeno grupo de indivíduos e sofreu uma grande perda de diversidade genética. Apesar de normalmente as populações introduzidas não apresentarem uma diminuição na sua variação genética quando são fundadas por um pequeno reduzido número de indivíduos, a probabilidade de apresentarem níveis de diversidade genética comparáveis com os da distribuição natural é extremamente reduzida. A distribuição do haplótipo invasor na América do Norte é praticamente restrita à região onde a subespécie *F. h. macrolepidotus* habita. Para além das populações do norte, este haplótipo foi encontrado em apenas dois indivíduos numa das populações do sul. Considerando a raridade com que foi encontrado no sul, e que é o haplótipo dominante na região norte, nós sugerimos que o mais

provável é que os indivíduos invasores sejam provenientes da região norte, entre Nova Iorque e Nova Escócia. No nosso caso, a baixa resolução espacial para deteção da população de origem deve-se essencialmente à falta de diferenciação genética entre populações invasores. Após comparação do padrão genético que seria esperado caso esta espécie tivesse sido introduzida através de água de lastro ou libertação de espécimes provenientes de aquariofilia, nós sugerimos que a segunda opção será o vetor de introdução mais provável. Água de lastro é um dos principais vetores de introdução de espécies invasoras, sendo capaz de transportar em simultâneo um grande número de organismos. Uma vez que existem inúmeras embarcações distribuídas globalmente, é provável que ocorram múltiplas introduções ao longo do tempo, cada uma composta por um grande número de indivíduos. Como grandes números de indivíduos tendem a transportar maior diversidade genética, seria expectável encontrar na Península Ibérica níveis de variação genética similares aos da distribuição nativa. Contudo, a falta de diversidade genética demonstrada pelos nossos resultados rejeita este cenário. Apesar do comércio de espécies ornamentais ser responsável por várias introduções a nível global, a libertação de baixos números de indivíduos de cada vez faz com que sejam necessários múltiplos eventos de introdução para que as populações invasoras não demonstrem um efeito de fundador. Adicionalmente, os peixes comercializados para aquariofilia tendem a estar no estado adulto ou apresentam uma boa condição física, o que os torna mais aptos a sobreviverem em ambiente natural após introdução. Acresce que *F. heteroclitus* é uma espécie extremamente resistente, pelo que a probabilidade de sobrevivência é ainda potencialmente maior. Por conseguinte, consideramos que a hipótese mais provável relativamente à introdução desta espécie na Península Ibérica, mais precisamente na costa sudoeste de Espanha, está relacionada com o comércio de espécies ornamentais. Por fim, evocamos três pontos que apoiam a hipótese de que o estabelecimento de uma população invasora no Delta do Ebro se deveu a uma introdução antropogénica posterior e não a colonização natural. Primeiramente, comparamos a estimativa do tempo decorrido durante a colonização natural da Ria Formosa com a estimativa do tempo decorrido num cenário hipotético de colonização natural do Ebro Delta. Considerando que esta espécie tem uma dispersão extremamente reduzida, e supondo que os indivíduos demoraram cerca de 20 anos para se deslocarem 50 km no caso de Faro e mais de 1000 km para chegarem ao Delta do Ebro, uma colonização de longa distância em tão pouco tempo é extremamente improvável. Em segundo lugar, num cenário de dispersão natural até ao Delta do Ebro seria expectável encontrar várias populações estabelecidas entre Cádiz, a localização mais a sul onde é encontrado da Península Ibérica, e o Delta do Ebro. Mais especificamente seria expectável encontrar esta espécie no Mar Menor, uma lagoa de água salgada de condições

favoráveis à colonização pelo *F. heteroclitus*, situada no Mediterrâneo. No entanto, como ainda não foram encontrados espécimes nesta lagoa, pensa-se que este local se encontra ainda por colonizar. Em último lugar, o estreito de Gibraltar não pode ser excluído como potencial barreira para a dispersão natural de *F. heteroclitus*. Apesar de ainda não ter sido descrito como barreira para esta espécie em concreto, sabe-se que o estreito de Gibraltar limita a distribuição de várias espécies, nomeadamente de peixes endémicos da Península Ibérica, *Aphanius iberus* e *Aphanius baeticus*, de ecologia semelhante a *F. heteroclitus*. Pelas razões acima mencionadas, sugerimos que introdução antropogénica a partir de indivíduos invasores previamente estabelecidos no sul da Península Ibérica é o cenário mais provável para explicar a presença de indivíduos no Delta do Ebro. Adicionalmente, considerando a grande tolerância a salinidade e temperatura por parte desta espécie, é extremamente provável que continue a expandir a sua distribuição geográfica, enquanto as condições enfrentadas nos habitats novos forem favoráveis ao seu estabelecimento.

Palavras-chave: DNA mitocondrial, citocromo b, espécies invasoras, vetor de introdução

## Abstract

Human activities such as trade and transport have increased considerably in the last decades, greatly facilitating the introduction and spread of non-native species at a global level. In the Iberian Peninsula, *Fundulus heteroclitus*, a small euryhaline coastal fish with short dispersal, was found for the first time in the mid-1970s. Since then, *F. heteroclitus* underwent range expansions, colonizing the southern region of Portugal, southwestern coast of Spain and the Ebro Delta in the Mediterranean Sea. We analysed mitochondrial DNA cytochrome *b* sequences to elucidate the invasion pathways in Iberia. We sampled three Iberian locations (Faro, Cádiz and Ebro Delta) and 13 other locations along *F. heteroclitus* native range in North America. Results revealed a single haplotype, common to all invasive locations, which can be traced to the northern region of the species native range. Thus, we suggest the origin of the founder individuals to be between New York and Nova Scotia. Additionally, the lack of genetic structure within Iberia is consistent with a recent invasion scenario and a strong founder effect. We suggest the most probable introduction vector is associated to aquarium trade and we further discuss the hypothesis of a second human mediated introduction responsible for the establishment of individuals in the Ebro Delta. Given the high tolerance to salinity and temperature, the species will most likely continue to spread geographically, as long as habitat requirements remain available.

Keywords: introduction vector, cytochrome *b*, mitochondrial DNA, invasive species



## Table of Contents

<b>AGRADECIMENTOS</b> .....	<b>I</b>
<b>RESUMO</b> .....	<b>II</b>
<b>ABSTRACT</b> .....	<b>V</b>
<b>TABLE OF CONTENTS</b> .....	<b>VI</b>
<b>INDEX OF FIGURES</b> .....	<b>VII</b>
<b>INDEX OF TABLES</b> .....	<b>VIII</b>
<b>LIST OF ABBREVIATIONS</b> .....	<b>IX</b>
<b>CHAPTER 1: INTRODUCTION</b> .....	<b>1</b>
1. MARINE BIOLOGICAL INVASIONS .....	1
1.1. INTRODUCTION VECTORS .....	2
1.2. ECOLOGICAL AND ECONOMIC THREATS .....	4
1.3. INVASIVE PATHWAYS .....	5
2. <i>FUNDULUS HETEROCLITUS</i> .....	6
2.1. BIOLOGY AND ECOLOGY OF <i>FUNDULUS HETEROCLITUS</i> .....	10
2.2. <i>FUNDULUS HETEROCLITUS</i> MISIDENTIFICATION.....	12
2.3. THE PRESENCE OF THE INVASIVE <i>FUNDULUS HETEROCLITUS</i> IN IBERIA .....	13
2.4. <i>FUNDULUS HETEROCLITUS</i> INTRODUCTION VECTOR .....	14
2.5. IMPACTS OF THE INVASIVE <i>FUNDULUS HETEROCLITUS</i> ON ENDEMIC BIODIVERSITY ...	15
3. OBJECTIVES .....	15
4. RESEARCH QUESTIONS AND HYPOTHESES.....	16
5. CONCLUSIONS .....	16
6. REFERENCES.....	17
<b>CHAPTER 2: INVASIVE GENETICS OF THE MUMMICHOG (<i>FUNDULUS HETEROCLITUS</i>): RECENT ANTHROPOGENIC INTRODUCTION IN IBERIA ....</b>	<b>26</b>
ABSTRACT .....	26
INTRODUCTION .....	27
MATERIALS AND METHODS.....	30
RESULTS .....	32
DISCUSSION.....	34
REFERENCES .....	39

## Index of Figures

### CHAPTER 1: INTRODUCTION

**Figure 1.1.** *Fundulus heteroclitus* current distribution. (A) Native distribution (red line) in the North American Atlantic coast. The morphological, physiological and genetic clines detected between northern and southern populations in North America are located between latitudes of 40 and 41° N black bold lines (*F. heteroclitus* photograph from: North American Native Fishes Association 2010). (B) Known invasive range (blue line and dot) in the Iberian Peninsula.....8

### CHAPTER 2: INVASIVE GENETICS OF THE MUMMICHOG (*FUNDULUS HETEROCLITUS*): recent anthropogenic introduction in Iberia

**Figure 2.1.** Figure 2.1. Distribution map showing the collection localities along the coast of North America (A), and Iberian Peninsula (B). Locations and sample details can be found in Table 2.1. Coloured circles display the distribution of the relative proportions of the cyt b haplotypes from each location: the four most common haplotypes, the private haplotypes, and all the other shared, but less frequent haplotypes, are represented (C, see legend).....31

**Figure 2.2.** Haplotype and nucleotide diversity from all sampled locations. Abbreviations in the legend are defined in Table 2.1 and colours are the same as in Figure 2.1.....33

**Figure 2.3.** Median-joining cytochrome *b* haplotype network for *Fundulus heteroclitus*. Each circle represents a different haplotype with size proportional to the frequency of the haplotype within the sample. Line length is proportional to the number of mutations between haplotypes. Each colour corresponds to a different location and are the same as in Figure 2.1. Locations details can be found in Table 2.1. Black dots represent putative unsampled haplotypes and the number in black square shows the number of mutations separating haplogroup A (A) from the haplogroup B (B).....34

## Index of Tables

### **CHAPTER 2: INVASIVE GENETICS OF THE MUMMICHOG (*FUNDULUS HETEROCLITUS*): recent anthropogenic introduction in Iberia**

**Table 2.1.** Sample location, sample abbreviations and summary statistics for a cytochrome *b* sequence fragment from *Fundulus heteroclitus*. ID refers to numbers in Figure 2.1.....30

## List of Abbreviations

bp – base pairs

cyt *b* – cytochrome b

*h* – haplotype diversity

ha – hectares

*Ldh-b* – lactate dehydrogenase-B

mtDNA – mitochondrial DNA

*n* – number of haplotypes

*n<sub>p</sub>* – number of private haplotypes

PCR – polymerase chain reaction

RFLP – restriction fragment length polymorphisms

SNPs – single nucleotide polymorphisms

TL – total length

$\pi$  – nucleotide diversity

## CHAPTER 1: Introduction

As a consequence of human activities involving large distance marine transportation and trade, the spreading of invasive species to new locations has been greatly facilitated (García-Llorente *et al.* 2008; Meyerson & Mooney 2007; Perrings *et al.* 2005; Rius *et al.* 2014; Williams *et al.* 2013). European marine biological invasion rates have increased dramatically in the last 50 years (Galil *et al.* 2014; Galil *et al.* 2016; Zenetos *et al.* 2012). According to Galil *et al.* (2014), there are over 850 invasive species in the European seas, of which 237 are found along the western European margins, 680 in the Mediterranean Sea and the remaining in the Baltic. Other studies make similar estimates, suggesting there are between 700 and 900 invasive species in the Mediterranean (Galil *et al.* 2016; Zenetos *et al.* 2012). The variation found between the estimates in the literature is probably due to the use of different definitions in each study. Here we refer to non-native or introduced species (synonyms: alien, exotic, non-indigenous, allochthonous) as “species, subspecies or lower taxon introduced intentionally or accidentally by a human-mediated vector outside its present or past natural range, and outside its natural dispersal potential” (Olenin *et al.* 2014). Additionally, we distinguish introduced from invasive species, defined as an “alien whose population has undergone an exponential growth stage and is rapidly extending its new range” (Occhipinti-Ambrogi & Galil 2004) and implies negative impacts on the recipient ecosystem (Russell & Blackburn 2017). Thus, we highlight the distinction between two processes: introduction or translocation and actual invasion, a possible but not obligatory outcome of the former (Blackburn *et al.* 2011; Russell & Blackburn 2017).

### 1. Marine biological invasions

According to an unified framework for biological invasions by Blackburn *et al.* (2011), the invasive process of a given species is composed by a sequence of four stages: transport, introduction, establishment and spread. Additionally, each stage is separated by different barriers which are required to be overcome for a species, or a population, to move on to the next stage (Blackburn *et al.* 2011). After overcoming the first barrier of geography via anthropogenic transport, a native species becomes non-native as it is introduced into the wild, in a new environment outside its natural distribution. However, not all non-native species are in fact introduced, since they might not be able to cross the human-made barriers imposed in the context of captivity and cultivation (barriers like fences). Nevertheless, two new barriers appear for the ones which manage to become introduced, preventing them from becoming

established: survival and reproduction. Non-native species must not only survive in the environmental conditions found in the new area, but also successfully reproduce. If the non-native species long-term population growth rate is not positive it might still go extinct and multiple introductions may be necessary before becoming effectively established. Finally, the dispersal and environmental barriers are the final obstacles preventing a non-native species from effectively spreading to new locations; it is only in this stage that a non-native species becomes invasive. The environmental barrier limits the extent to which the invasive species expand their range by offering potentially adverse environmental conditions which are inappropriate for successful colonization of new areas. When a given species manages to overcome all barriers, the result is a fully invasive species, capable of dispersing, surviving and reproducing at several locations across a greater or lower scale (Blackburn *et al.* 2011).

Marine biological invasions have three main areas of concern: the vectors of invasions, that is the means by which the individuals expand their distribution to non-native areas, the threats that a new species will inflict on the existing dynamic equilibrium and the pathway of invasion, which can assist in predicting how the invasive species will geographically spread. Each of these points will be taken into consideration next, building an introductory overview of the subjects.

## **1.1. Introduction vectors**

Invasive species spread and occupy new marine and coastal ecosystems through several maritime introduction vectors, which are the “physical means or agent by which a species is transported” (Carlton 2001a). The main marine introduction vectors are ballast water, biofouling of vessel hulls, aquaculture, aquarium trade, live bait, live seafood trade and marine debris (Williams *et al.* 2013).

Vessels can transport various invasive species via ballast water and biofouling. Ballast water is pumped into ships to provide them with stability and to compensate for the lack of cargo (Carlton 2001a). However, indiscriminate water pumping results in accidentally loading a wide range of species (Briski *et al.* 2012; Carlton 2001a, b; Gollasch 2007). Although the individuals transported are usually extremely small, such as plankton or other viable resting stages present within the ballast tank sediment (Briski *et al.* 2012; Carlton 2001a; Carver & Mallet 2002; Padilla & Williams 2004), introduction of bigger species, such as the Round Goby (*Neogobius melanostomus*) has been linked to ballast water (Fuller *et al.* 2017). Furthermore, as vessels and other submerged infrastructures are extremely prone to be colonized by a diverse

community of sessile and some mobile organisms, extensive biofouling has been demonstrated in hulls, sea-chests, anchors, pipes or rigs (Bax *et al.* 2003; Coutts & Dodgshun 2007; Davidson *et al.* 2009; Mineur *et al.* 2008; Wanless *et al.* 2010). Thus, moving these colonized surfaces provides a unique opportunity of human-mediated dispersal to species which could not survive natural long-distance drifting (Carlton 2001a).

Accidental and intentional release of exotic species from aquaculture facilities is another important introduction vector. Since many farmed species, such as fishes and bivalves, are non-native relatively to where they are being grown, and that specimens are frequently entering and leaving the facilities, aquacultures are especially prone to being responsible for multiple and frequent introductions (Bax *et al.* 2003; Naylor *et al.* 2001; Rius *et al.* 2014). Moreover, aquarium trade can be also associated to both intentional and accidental release of invasive species. For instance, fish may be released when they become unwanted by their owners (Duggan *et al.* 2006; Padilla & Williams 2004). Introductions may also be attributed to accidental escape from tanks during storms or to the release of water containing non-native organisms from domestic tanks and public aquariums (Duggan *et al.* 2006; Padilla & Williams 2004; Rhyne *et al.* 2012). Considering that some species are commercialized at a global level and are extremely widespread, they can colonize all freshwater and marine environments (Padilla & Williams 2004). Similarly, the live seafood trade transports mature individuals at a global scale. Thus, the probability of some viable individuals reaching a natural environment and becoming established can be high (Chapman *et al.* 2003).

The use of non-native live bait by anglers is also seen as an important introduction vector of invasive species, given unused live bait is commonly released into the environment where organisms may become established (Carlton 2001a; Kilian *et al.* 2012). Lastly, in the last five decades, the huge increase of anthropogenic floating marine litter in environment, namely plastic debris, has provided a significant alternative mean of transportation for several rafting species, which used to rely solemnly on natural floating materials for the dispersal of reproductive propagules (Katsanevakis & Crocetta 2014). Floating debris can be colonized by a wide range of taxa, which may spread and establish in new areas if conditions are favourable (Carlton 2001a; Katsanevakis & Crocetta 2014 and references therein).

Regarding the European Seas, Galil *et al.* (2014) referred to shipping and culture activities as relatively more important in the western European margins and in the Baltic, whereas in the Mediterranean Sea the Suez Canal, a human-made corridor linking the Red Sea to the Mediterranean, is responsible for roughly more than half of the non-native species introduced. Likewise, Gollasch (2007) had previously reported the three most important vectors in Europe

were ballast-water, followed by fouling and aquaculture. However, if one focuses on the Mediterranean individually, it becomes clear that although roughly half of the invasive species were introduced via the Suez Canal, the relative importance of the vectors along its area is not homogeneous: shipping and aquaculture have a stronger impact on the western Mediterranean, whereas The Suez Canal is responsible for most invasions on the eastern Mediterranean (Galil *et al.* 2016; Zenetos *et al.* 2012).

## **1.2. Ecological and economic threats**

Marine invasive species pose a significant environmental threat since biological invasions are one of the major drivers of biodiversity loss, the others being habitat change, climate change, overexploitation and pollution (Brook *et al.* 2008; Millennium Ecosystem Assessment 2005). Marine invasive species are capable of deeply altering the invaded ecosystems by shifting nutrient and sediment dynamics, changing the available light, modifying the community structure and the trophic food web, causing the loss of native genotypes and displacing native species (Bax *et al.* 2003; Molnar *et al.* 2008; Wallentinus & Nyberg 2007). Hybridization, for instance, may lead to the loss of native genotypes via introgression of invasive genes into the native gene pool. The resultant hybrids may outcompete the native species (Grosholz 2002; Hänfling 2007; Vilà *et al.* 2010): for example, Rosenfield *et al.* (2004) demonstrated the hybrids original from the crossing of invasive sheepshead minnow (*Cyprinodon variegatus*) with the endemic Pecos pupfish (*Cyprinodon pecosensis*) in New Mexico, USA, displaced the endemic species due to their higher fitness.

Biological invasions are also responsible for substantial economic losses which can arise from decreased fisheries and aquaculture production, extensive fouling of vessels' hulls and other marine infrastructures such as water pipes (Bax *et al.* 2003; Molnar *et al.* 2008). For instance, the zebra mussel (*Dreissena polymorpha*), a Ponto-Caspian freshwater bivalve which has extensively invaded North America, is a good example of a widespread invasive species with multiple impacts (e.g., Connelly *et al.* 2007; Strayer 2009). Strayer (2009) reviewed an extensive list of ecological and economic impacts caused by its widespread invasion, since they provoked deep changes in the community structure and ecosystem dynamics. High densities of zebra mussels change nutrient concentrations, available light and phytoplankton biomass, leading not only to a decrease in the biomass of pelagic fish, but also to the extinction of native bivalves which are outcompeted by this invasive species (Strayer 2009). Moreover, zebra mussels are known for fouling water intake pipes and other infrastructures in electric power generation and drinking water treatment facilities, greatly reducing the water inflow. When this



happens, industries must undertake remediation measures along with prevention plans to avoid future infestations, both of which entail significant maintenance costs (Connelly *et al.* 2007). In another instance, the ctenophore *Mnemiopsis leidyi*, a gelatinous predator, is an example of ecosystem changes and consequent economic impacts after establishment. *Mnemiopsis leidyi* was introduced in the Black Sea in the early 1980s, probably via ballast-water (Shiganova 1998). During 1989, increased zooplankton biomass due to overfishing in previous years allowed explosive growth of the invasive *M. leidyi* (Shiganova 1998). The intensive predation on eggs and larvae of fish that followed the bloom collapsed the fisheries of planktivorous fish mainly in the northern region of the Black Sea, an area already under severe anthropogenic damage (Shiganova 1998).

Invasive species may also increase the frequency of harmful algal blooms or foment the spread of disease-causing viruses and bacteria, directly threatening human health (Ruiz *et al.* 1997). Finally, increased disturbance in a certain environment due to the negative impacts caused by invasive species may facilitate new future invasions, potentially culminating in an “invasional meltdown” (Adams *et al.* 2003; Simberloff & Von Holle 1999).

### **1.3. Invasive pathways**

After becoming established, it is almost impossible to completely eradicate invasive species (Katsanevakis & Crocetta 2014); eradication measures may be effective in the early phases of invasion but they are costly, require considerable effort and are often unsuccessful (Katsanevakis & Crocetta 2014; Otero *et al.* 2013). Thereby, preventing the establishment of new invasive species is of extreme importance and probably the best way to avoid the negative impacts associated to the spread of non-native species (Katsanevakis & Crocetta 2014; Otero *et al.* 2013). However, management at the introduction vector level is the only effective way to prevent new primary or secondary introductions (Katsanevakis & Crocetta 2014).

Alternatively, a deep understanding of the invasion pathway, which is the route that a non-native species took to invade in a new region (Olenin *et al.* 2014), is crucial not only to prevent new invasions, but also to aid the development of effective conservation measures (Galil *et al.* 2014; Ghabooli *et al.* 2013; Handley *et al.* 2011). For instance, information on the ecology of the invasive species within its native range may help choosing adequate biological control agents for containment measures (Roderick & Navajas 2003). Furthermore, knowledge of the introduction vectors, population sources and their genetic characteristics can be used to create risk maps, which provide useful insights into the potential ecological impacts (Kulhanek

*et al.* 2011). Comparative risk analysis can be used more specifically to predict the impacts of different routes and the probability of occurring new primary or secondary invasions based on environmental suitability (Hulme 2009; Molnar *et al.* 2008; Stepien *et al.* 2005).

However, it is a difficult task to reconstruct the invasion pathway and identify the putative source populations with historical and contemporary vector records, since they are frequently incomplete and misleading (Estoup & Guillemaud 2010; Handley *et al.* 2011). In this context, molecular genetic data is a powerful tool to recreate the invasive history: it identifies putative source populations and genetic bottlenecks, distinguishes between single or multiple introductions and discriminates between primary or secondary invasions (Bock *et al.* 2015; Cristescu 2015; Estoup & Guillemaud 2010; Handley *et al.* 2011). Although genetic data does not always allow for successful identification of these processes, there are three main conditions which increase the probability of accurate reconstruction of the invasive pathway, all fulfilled for *Fundulus heteroclitus*: (1) extensive sampling along the native range to ensure all the putative source populations and all native haplotypes are known, (2) presence of genetic structure in the native range to narrow down the putative source regions and (3) a short amount of time elapsed since the invasion, so that processes such as genetic drift do not increase the genetic differentiation between native and invasive populations (Cristescu 2015; Geller *et al.* 2010).

## **2. *Fundulus heteroclitus***

The biology and ecology of the mummichog, *Fundulus heteroclitus* (Linnaeus, 1766), have been extensively studied (e.g., Burnett *et al.* 2007). It was referred as a “textbook example” of intraspecific latitudinal genetic variation by Avise (2004), being an important model for adaptive genetic cline studies due to its abundance, easy manipulation and unusually high level of polymorphism (e.g., Cashon *et al.* 1981; Nevo 1976; Powers & Place 1978; Van Beneden *et al.* 1981; Whitt 1969). *Fundulus heteroclitus* exhibits distinct and extensive latitudinal clinal variation in relation to morphological, physiological and genetic traits along the North American Atlantic coast, which have been thoroughly studied.

Geographical analysis of morphological traits (Able & Felley 1986; Morin & Able 1983), enzyme-coding loci (Powers & Place 1978; Powers *et al.* 1986; Ropson *et al.* 1990), mitochondrial DNA (Bernardi *et al.* 1993; González-Vilaseñor & Powers 1990; Smith *et al.* 1998), putative neutral nuclear microsatellite loci (Adams *et al.* 2006; Duvernell *et al.* 2008), and a variety of nuclear single-nucleotide polymorphisms (McKenzie *et al.* 2015, 2016; Strand

*et al.* 2012; Williams *et al.* 2010) demonstrated concordance of clinal patterns, which are centred between 40° and 41° N along the coast of New Jersey, USA. These studies concur with Morin & Able (1983), supporting the division of *F. heteroclitus* in two subspecies: *F. heteroclitus heteroclitus* (Linnaeus, 1766) in the locations below the cline (referred to as the southern populations) and *F. heteroclitus macrolepidotus* (Walbaum, 1792) above the cline (referred to as the northern populations).

Current genetic patterns, characterized by restricted gene flow and isolation by distance between northern and southern populations, already existed before the last glacial period as the range expansion following glacier retraction did not disrupt them (Adams *et al.* 2006; Haney *et al.* 2009). Adams *et al.* (2006) supported previous studies (e.g., González-Vilaseñor & Powers 1990; Ropson *et al.* 1990) by reporting the establishment of a secondary contact zone 15,000 years ago during the last glacial retreat, after the sea level increase connected the once separated northern and southern populations. At the time, the Hudson River (40-41° N, Figure 1.1) presented itself as barrier to dispersal (Duvernell *et al.* 2008), especially while the glaciers were retreating and its flow increased considerably with the meltwater (Donnelly *et al.* 2005). Today, the secondary contact zone maintains and corresponds to the latitudes of clinal variation (Brown & Chapman 1991; Powers *et al.* 1991).

Cashon *et al.* (1981) stated that the cline location coincides with the southernmost extent of the ice sheet at the time of maximum glaciation, implying that the northern saltmarshes were destroyed under the ice sheet, while the location of the southern saltmarshes varied due to sea level fluctuations. Thus, they suggested that the distribution of the current genetic patterns was shaped by the last glacial event 20,000 years ago (Cashon *et al.* 1981). González-Vilaseñor & Powers (1990) estimated the date of divergence between the two subspecies to have occurred one million years ago. Although this excludes the last glacial event as the trigger for genetic divergence, the last glacial events were probably responsible for shaping *F. heteroclitus* present-day distribution, being responsible for several episodes of population expansion and contraction (Ropson *et al.* 1990; Smith *et al.* 1998).

Northern populations would have been best positioned to recolonize newly available habitats during glacial retraction, which were best suitable for species already adapted to colder temperatures (Powers & Schulte 1998; Ropson *et al.* 1990). Both microsatellite and mitochondrial DNA (mtDNA) data suggest that some of the northern populations managed to persist in the glaciated areas, although their distribution was patchy, discontinuous and with restricted gene flow (Adams *et al.* 2006; Haney *et al.* 2009). Furthermore, the estimated date of establishment of the two northernmost populations which should be the most recent ones,

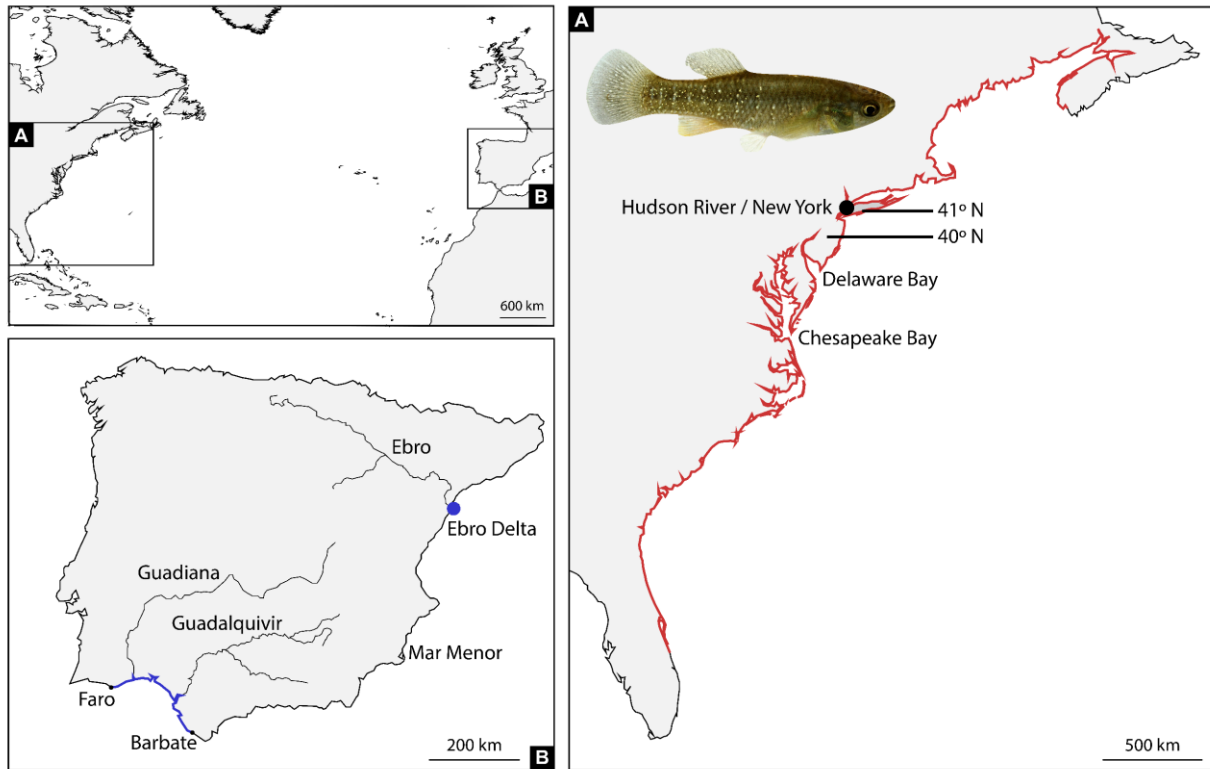


Figure 1.1. *Fundulus heteroclitus* current distribution. (A) Native distribution (red line) in the North American Atlantic coast. The morphological, physiological and genetic clines detected between northern and southern populations in North America are located between latitudes of 40 and 41° N black bold lines (*F. heteroclitus* photograph from: North American Native Fishes Association 2010). (B) Known invasive range (blue line and dot) in the Iberian Peninsula.

matches a period of time between the beginning and the end of the last glacial maximum (Haney *et al.* 2009). This estimate suggests a range expansion within the glaciated area, which might have already started before the end of the last glacial event (Haney *et al.* 2009). Populations with the northern haplotype can also be found south of the contact zone, in the areas of extreme freshwater upper estuary (Smith *et al.* 1998). Their presence in such environments was probably a result of either a northward expansion of the northern haplotypes as the glaciers retreated, or of selection, as the northern haplotypes are better adapted to freshwater environments (Scott *et al.* 2004), potentially outcompeting the less fit southern haplotype (Smith *et al.* 1998; Whitehead 2009).

After historical separation, clines may arise when the barrier to dispersal is removed, and gene flow and migration start occurring between populations (Powers & Place 1978). The cline formed in the contact zone will erode with gene flow (Brown & Chapman 1991), depending on distance and frequency of gene flow (Endler 1977). In the absence of selection, *F. heteroclitus* dispersal must be less than one or two km to maintain its allozyme clines located in the contact zone, which remain stable up to day (Brown & Chapman 1991). Their

results are congruent with previous reports of *F. heteroclitus* displaying short dispersal distances (1-2 km, Fritz *et al.* 1975; Lotrich 1975). Although clines may have arisen due to isolation, they are currently maintained by natural selection which may vary both in type and intensity in different loci: not all clines are located exactly at 40° N (Brown & Chapman 1991). Location discrepancies found among the clines for mitochondrial DNA and individual nuclear genes suggest selection has been impacting the geographical variation of some loci, whereas others are more affected by stochastic random processes (Ropson *et al.* 1990).

According to Ropson *et al.* (1990), selection is commonly used to explain clines found in morphological traits and polymorphic proteins, which are linked to an environmental gradient, such as mean water temperature. In this case, clines are located along a steep thermal gradient, where temperature is correlated with latitude (Fangue *et al.* 2006; Place & Powers 1979; Powers & Place 1978). In this context, thermal selection has been suggested to be responsible for maintaining these clines (reviewed in Powers & Schulte 1998; Schulte 2001): for example, biochemical data on the lactate dehydrogenase-B (*Ldh-B*) allozyme has shown selective advantage in warmer or colder habitats depending on the genotype (Place & Powers 1977; Powers 1972; Powers & Powers 1975). The northern populations of *F. h. macrolepidotus* are associated to the *Ldh-B<sup>b</sup>* genotype which displays a selective advantage in cold temperatures, while the southern populations are associated to the *Ldh-B<sup>a</sup>* genotype, better adapted to higher temperatures (DiMichele & Powers 1982; Powers & Place 1978; Powers & Schulte 1998; Schulte 2001). The presence of genetic patterns like the ones created by differential fitness of *F. heteroclitus* genotypes in relation to water temperature are also found in other species which exhibit thermal selection in clinal maintenance, supporting this theory (Powers *et al.* 1986). Selection against warm water phenotypes in other nonmigratory polymorphic species, which recolonized the once glaciated northern areas during the last glacial event retreat has been already demonstrated (e.g., Christiansen & Frydenberg 1974; Corbin 1977). More recently, McKenzie *et al.* (2015) suggested the secondary contact area corresponds to a bimodal hybrid zone, which presents some level of pre- and/or post-zygotic reproductive isolation influencing its population structure, even though isolation is not complete as there is still some gene flow occurring between the subspecies; strong selection may be acting against less fit hybrids who exhibit maladapted combinations of distinct parental genotypes.

## 2.1. Biology and ecology of *Fundulus heteroclitus*

This ubiquitous killifish can be found in freshwater, brackish or saltwater, and inhabits sheltered coastal areas such as saltmarshes, tidal creeks, estuaries or bays all year-round (Bigelow & Schroeder 1953; Hardy Jr 1978). *Fundulus heteroclitus* withstands a wide range of salinities, from 0 to 120.3 ppm (Griffith 1974), and temperatures, from -1.5 °C (Umminger 1972) to 36.3 °C (Garside & Chin-Yuen-Kee 1972), surviving abrupt changes in both parameters (Bulger 1984; Hardy Jr 1978). In North America, its size, among other characteristics, is correlated with latitude: total length (TL) can vary from 90 mm in the south, up to 100-120 mm at the most northern areas of its distribution (Kneib & Stiven 1978). In the Iberian Peninsula, fish caught in the Guadalquivir marshes had sizes between 100-120 mm (TL, up to 132 mm, Fernández-Delgado 1989) while fish from the Ebro Delta were generally slightly smaller (TL, up to 104 mm, Gisbert & López 2007).

With a diet mainly composed of benthic invertebrates from the vegetated intertidal saltmarsh, such as small crustaceans and annelids (Baker-Dittus 1978; Kneib 1986, 1997; Kneib & Stiven 1978; Prinslow *et al.* 1974), *F. heteroclitus* is an opportunistic carnivorous species (Kneib 1986; Smith *et al.* 2002; Valiela *et al.* 1977). Its diet composition reflects prey availability as they appear inside the digestive tracts in proportions similar to their occurrence in the environment (Baker-Dittus 1978; Kneib 1986). Furthermore, prey selection and ingestion are size-dependent, as fish with higher dimensions are capable of ingesting bigger preys that smaller fish cannot (Baker-Dittus 1978; Vince *et al.* 1976). Along with the small invertebrates, fish also ingest detritus and plant particles, which can be found in large amounts in their stomachs; in the past, these elements were considered to be part of their diet (Jeffries 1972). Following this line of thought, Kneib & Stiven (1978) made a clear distinction between individuals smaller than 30 mm which were considered carnivorous, and the ones with larger dimensions capable of ingesting bigger plant particles which were thought to be omnivorous. However, the nutritive value of detritus and plant particles was proved to be very low, and thus, irrelevant to assure their growth or even to maintain their weight (Prinslow *et al.* 1974).

Unlike many other fish species, *F. heteroclitus* life history is closely linked to the intertidal saltmarsh (Kneib 1984). This is extremely clear when we consider, for instance, the movements related to their feeding habits. Feeding events are connected to the tides, where ingestion of a wider range of preys occurs at high tide at the surface of the saltmarsh vegetated areas, followed by a return to the tidal creeks as the tide goes out (Butner & Brattstrom 1960; Kneib & Stiven 1978; Teo & Able 2003; Weisberg *et al.* 1981). Butner & Brattstrom (1960)

reported that *F. heteroclitus* individuals presented empty guts when moving onto the upper vegetated areas of the saltmarsh and full guts when returning to tidal creeks at low tide. *Fundulus heteroclitus* presents a diurnal feeding chronology overlapping with the tidal rhythm benefitting from the wide range of available prey at the surface during high tide (Weisberg *et al.* 1981). This feeding pattern might not be directly connected to the preys, because when the high tides are not sufficient to flood the saltmarsh surface, fish still feed at the lower portions of the saltmarsh (Weisberg *et al.* 1981). Thereby, even though feeding in the subtidal is not enough for maintaining population growth at normal densities (Kneib 1986; Weisberg & Lotrich 1982), feeding events might be triggered by water volume variation or chemical changes linked to the high tides, rather than being correlated to prey abundance (Weisberg *et al.* 1981).

Considering that these tide-dependent movements to the higher parts of the saltmarsh are recognized as an adaptive trait in the life cycle of marine and estuarine species (Taylor *et al.* 1979), it is not surprising that this behaviour in *F. heteroclitus* has also been described related to their reproductive strategies (e.g., Able & Castagna 1975; Taylor *et al.* 1977). Populations from Delaware Bay (Figure 1.1) have been observed moving to the marsh surface during night high tide at the new or full moon spring tides, where the eggs were deposited on the inner surface of the primary leaves of *Spartina alterniflora*, resulting in exposure to desiccation during their development until a new high tide submerged them again (Taylor *et al.* 1977). *Fundulus heteroclitus* eggs can also be found in intertidal empty shells of the Atlantic Ribbed Mussel *Geukensia demissa* (= *Modiolus demissus*) which are exposed to periods of desiccation and high temperatures for several hours during low tides (e.g., Able & Castagna 1975; Taylor & DiMichele 1983). These complex semi-lunar reproductive behaviours not only increase the survival of the eggs by avoiding predation during the early-stage development, but also decrease the probability of water movements dislodging them to unfavourable locations, reducing the potential dispersal of the eggs, but increasing the survival probability (Taylor *et al.* 1977; Teo & Able 2003).

Unlike adult mummichogs which return to the tidal creeks, the young often remain at the saltmarsh surface after the tide recedes, concentrating in residual tidal water pools (Kneib 1984, 1986) up to 6-8 weeks after hatching (Taylor *et al.* 1979). Not only it seems to be advantageous in terms of refugia from predators as the areas are too shallow for eventual predators (Kneib 1984, 1987), but also in terms of higher prey abundance throughout the day (Kneib 1984). In this case, high densities of young mummichogs might have a strong impact on prey abundance, until the next inundating high tide restores natural prey densities (Kneib 1986; Kneib & Stiven

1978). This specific behaviour by the young allows one to discount the dispersal during early life phases, as it dramatically reduces the dispersal distance (Kneib 1986). Both the adhesive properties of the eggs and the fact that they remain in small tidal pools during low tides, reduce greatly the risk of being dislodged and dragged to unfavourable locations by strong currents.

All the movements made between the saltmarsh surface and the tidal creeks for foraging, spawning and predator avoidance described above may suggest considerable mobility and dispersal, but in fact this species home ranges are relatively short. Bigelow & Schroeder (1953) referred to it as one of the most stationary marine species. Chidester (1920) reported that *F. heteroclitus* remained in the tidal creeks throughout the summer while other species moved in and out with the tides, and in autumn fish moved into deeper, more saline waters. In a study about the species home range during the summer months, Lotrich (1975) observed a range of 36-38 m in individuals larger than six cm, indicating that most fish remained near the traps throughout the study. However, Fritz *et al.* (1975) reported that individuals travelled up to two km upstream during fall and winter months, despite also stating *F. heteroclitus* may be polytypic in its winter behaviour as the results do not concur with Chidester (1920). Sweeney *et al.* (1998) found out they moved up to 650 m from the marking site and Teo & Able (2003) calculated a home range of 15 hectares (ha), which corresponds to a linear distance of around 400 m. Skinner *et al.* (2005) reported 96.6% of the mummichogs were recaptured within 200 m of the original point of release. Additionally, even though their results come from allozyme frequency analysis, Brown & Chapman (1991) suggested that the *F. heteroclitus* mean dispersal distance at the Chesapeake Bay (Figure 1.1.) must be less than one or two km to maintain the allozyme frequency clines in the absence of strong selection. Teo & Able (2003) suggested that the variance among home range values from different studies may be due to differences in the sampling methods employed which, for instance, may act as a filter trapping fish with a certain range of lengths, or due to habitat differences where distances between the saltmarsh surface and the tidal creeks vary considerably. Nevertheless, the results mentioned clearly indicate *F. heteroclitus* as species with high site fidelity and short dispersal.

## **2.2. *Fundulus heteroclitus* misidentification**

Before being correctly identified as *F. heteroclitus*, specimens collected in the past were misidentified as *Valencia hispanica* (Valenciannes, 1846) and *Valencia lozanoi* (Gómez, Peiró & Sánchez, 1984) for almost 15 years. Machado (1857) reported for the first time the presence of *V. hispanica* in the southwestern Iberian Peninsula coast in the Guadalquivir saltmarshes.



Decades later, its presence in marshes in Spain (Guadalquivir) and in Portugal (Guadiana) was confirmed by Hernando (1975) and Coelho *et al.* (1976) respectively. Years later, Gomez-Caruana *et al.* (1984), after comparing the morphology of the *V. hispanica* provenient from Cádiz and Valencia, described the population from the lower Guadalquivir as a new species, *Valencia lozanoi*, based on their distinct morphologies and large discontinuity between the two sites. The appearance of these new species in the literature was followed by controversy regarding these populations taxonomic status. Considering their morphological characteristics, Fernández-Delgado *et al.* (1986), supported by Bianco & Miller (1989), suggested that the populations of *V. hispanica* previously identified by Hernando (1975), Coelho *et al.* (1976) and Arias & Drake (1986), and the populations of *V. lozanoi* described by Gomez-Caruana *et al.* (1984) were in fact members of *F. heteroclitus*. However, considering *F. heteroclitus* was probably introduced in the southwestern coast of Spain in the early 1970s and that the report by Machado (1857) was valid, *V. hispanica* should have been present in this area until the end of the 19<sup>th</sup> century, where it probably went extinct long before the *F. heteroclitus* first Iberian records (Planelles & Reyna 1996).

Mitochondrial DNA analysis of specimens from the Guadalquivir River (Lebrija, Sevilla) confirmed that these individuals were members of *F. heteroclitus* supporting what other authors had previously concluded based on morphology (Bernardi *et al.* 1995; Fernández-Pedrosa *et al.* 1996), including the renaming of *V. lozanoi* (Fernández-Delgado *et al.* 1986). Comparison of samples of *F. heteroclitus* from the Guadalquivir with *V. hispanica* from Valencia demonstrated clear distinctions between these two species (Fernández-Pedrosa *et al.* 1996). Furthermore, Bernardi *et al.* (1995) suggested the *F. heteroclitus* from the Guadalquivir marshes corresponded to the northern populations of *F. heteroclitus* from North America, supported by Fernández-Pedrosa *et al.* (1996) who confirmed most of individuals presented an Iberian haplotype correspondent to the *F. heteroclitus macrolepidotus* haplotype previously described in the literature (Brown & Chapman 1991; González-Vilaseñor & Powers 1990).

### **2.3. The presence of the invasive *Fundulus heteroclitus* in Iberia**

Recently, *Fundulus heteroclitus* was found across the Atlantic in the Iberian Peninsula, which suggests a human-mediated introduction. Though the date of introduction in the southern coast Spanish saltmarshes remains uncertain, it was probably introduced between 1970 and 1973 (Fernández-Delgado 1989). Although Gutiérrez-Estrada *et al.* (1998) suggested some limitations (see below), they did not exclude the early 1970s as the most likely date of

introduction. Almaça (1995) had no suggestion regarding the date of introduction of *F. heteroclitus* in the Portuguese side of the Guadiana saltmarshes because fish research at the mouth of the Guadiana only took place after 1975, and thus it could have been present for a long time in this region without being reported. By the 1990s it was already well-established in the southwestern coast of Spain, where it could be found almost continuously from the mouth of the Guadiana until the Barbate marshes (Gutiérrez-Estrada *et al.* 1998). A decade later, its presence was recorded in the Ria Formosa, southern coast of Portugal (at least since 2002 in seabird pellets; e.g., Catry *et al.* 2006; Paiva *et al.* 2006b) and in the Ebro Delta in the Mediterranean Sea, northeastern coast of Spain (Gisbert & López 2007) (Figure 1.1).

#### **2.4. *Fundulus heteroclitus* introduction vector**

Despite having been introduced in the Iberian Peninsula four decades ago, consensus on the introduction vector responsible for its establishment in the southwestern coast of Iberia is yet to be reached. Fernández-Delgado (1989) suggested it was introduced when two other American species were intentionally introduced in this area: the large-mouth bass (*Micropterus salmoides* Lacépède) and the crayfish (*Procambarus clarki* Girard). However, Gutiérrez-Estrada *et al.* (1998) concluded that *F. heteroclitus* introduction continued unknown and could have not been associated to the crayfish. The latter was only introduced in the Guadalquivir marshes in 1974 (Delibes & Adrián 1987) while adult mummichogs were only collected from the Guadalquivir marshes in March 1973 (Hernando 1975). Bernardi *et al.* (1995) also suggested *F. heteroclitus* was introduced involuntarily without providing further hypotheses. Furthermore, its introduction has been linked either to aquarium trade (Elvira & Almodóvar 2001; García-Llorente *et al.* 2008; Gozlan 2010; Hernando & Soriguer 1992; Ribeiro *et al.* 2008a) or ballast-water (Fernández-Delgado 2010; García-Revilla & Fernández-Delgado 2009). Additionally, Gozlan (2010) suggested the human-mediated intentional introduction could be related not only to aquarium trade but also to biological control.

Furthermore, Gisbert & López (2007) proposed a human-mediated secondary invasion hypothesis to explain *F. heteroclitus* more recent introduction in the Ebro Delta. They suggested establishment in the Ebro happened after live fish were transported by road from the southwestern Iberian region, to be used in aquaculture and aquariology purposes, as well as in scientific studies. It is known that the Aquaculture Research Centre of the Institute for Food and Agriculture (IRTA) located in the Ebro Delta used *F. heteroclitus* as a biological model between 2001 and 2004, undertaking measures to avoid *F. heteroclitus* accidental escape at all

life stages (Gisbert & López 2007). However, considering that individuals were found in the vicinity of the research facilities (ca. 2 km away) and that accidental escape has been responsible for the spread of other exotic species in the Ebro Delta (e.g., Oriental Weather Loach Franch *et al.* 2008; Maceda-Veiga 2013), this hypothesis cannot be ruled out (Gisbert & López 2007).

## **2.5. Impacts of the invasive *Fundulus heteroclitus* on endemic biodiversity**

In the Iberian Peninsula, successful introductions of exotic species such as the mummichog *F. heteroclitus* and the eastern mosquitofish *Gambusia holbrooki* (Agassiz, 1859) may have a negative impact on the coexistent endemics (e.g., Elvira & Almodóvar 2001). The euryhaline *F. heteroclitus* inhabits shallow coastal areas alongside with other small endemic toothcarps, such as the *Aphanius iberus* (Valenciennes, 1846) and *V. hispanica* in the Mediterranean coast of Spain, and the *Aphanius baeticus* in the southwestern Spanish coast (Doadrio, Carmona & Fernández-Delgado, 2002). *Aphanius iberus* distribution is restricted to eight populations in the southwestern Atlantic coast of Spain (Doadrio *et al.* 2002). Cyprinodontid fish may achieve high population densities, hampering a healthy coexistence with other species (Clavero *et al.* 2007) and toothcarp densities are subject to a great decrease when they share their habitat with exotic invasive species like *F. heteroclitus* or *G. holbrooki* (Clavero *et al.* 2007; Fernández-Delgado *et al.* 1999). *Fundulus heteroclitus* poses a potential threat by competition and/or predation of the endemic species, and may act synergistically with habitat destruction resulting in a more profound negative impact (Bernardi *et al.* 1995; Doadrio *et al.* 2002; Elvira 1996; Elvira & Almodóvar 2001; Fernández-Delgado 1989; García-Berthou *et al.* 2007; García-Llorente *et al.* 2008; Leunda 2010; Oliva-Paterna *et al.* 2006; Planelles & Reyna 1996). Currently, both species of Iberian *Aphanius* are ranked as endangered (Crivelli 2006a, b) and *V. hispanica* is ranked as critically endangered (Crivelli 2006c).

## **3. Objectives**

In this study, we aim to unravel the Iberian invasion history of the mummichog (*Fundulus heteroclitus*), in terms of both geographic origins, genetic and demographic processes based on a fragment of the mitochondrial DNA cytochrome *b* gene. Samples covering most of the current native distribution of the species were included to infer colonization pathways.

#### 4. Research questions and hypotheses

Have the *Fundulus heteroclitus* populations from Iberia experienced a founder effect upon invasion?

Where are the invasive Iberian populations original from?

What is the genetic structure within the *F. heteroclitus* populations of the Iberian Peninsula?

Was the range expansion in Iberia due to natural dispersal?

Which are the most probable introduction vectors for the invasion of *F. heteroclitus* in Iberia?

Given that *F. heteroclitus* was first reported in the Iberian Peninsula quite recently (in the early 1970s, Fernández-Delgado 1989) and is a non-migratory species with limited dispersal (less than 2 km, Fritz *et al.* 1975; Lotrich 1975), we posit the following hypotheses:

- (1) If the recent human-mediated introduction and subsequent invasion of the mummichog in Iberia originated on a few founder individuals, then the genetic diversity of the Iberian populations must be low and the haplotypes identified in the Iberian populations will be traceable to one or multiple locations in North America, identifying the possible population sources;
- (2) If the species in Iberia has low genetic diversity and given the short time since the invasion, populations are not expected to exhibit high genetic structure;
- (3) If the dispersal between locations was not human-mediated and followed local currents, the distribution and frequency of haplotypes will reflect the invasion pathway.

#### 5. Conclusions

We propose that the genetic diversity and structure from the Iberian *F. heteroclitus* can be explained by a single introduction event carrying a very small number of individuals sharing the same haplotype. Furthermore, aquarium trade of *F. heteroclitus* individuals from their native northern range between New York and Nova Scotia seems the most plausible introduction vector in Iberia populations. Moreover, we discuss that the *F. heteroclitus* individuals found in the Ebro Delta became established following a secondary human-mediated introduction event with individuals from the south of the Iberian Peninsula. Considering the high tolerance to salinity and temperature and the significant amount of colonized area in the Iberian southern region, the species will most likely keep on expanding its invasive range until it faces unfavourable environmental conditions.

## 6. References

- Able KW, Castagna M (1975) Aspects of an undescribed reproductive behavior in *Fundulus heteroclitus* (Pisces: Cyprinodontae) from Virginia. *Chesapeake Science* **16**, 282-284.
- Able KW, Felley JD (1986) Geographical variation in *Fundulus heteroclitus*: tests for concordance between egg and adult morphologies. *American Zoologist* **26**, 145-157.
- Adams MJ, Pearl CA, Bruce Bury R (2003) Indirect facilitation of an anuran invasion by non-native fishes. *Ecology Letters* **6**, 343-351.
- Adams SM, Lindmeier JB, Duvernell DD (2006) Microsatellite analysis of the phylogeography, Pleistocene history and secondary contact hypotheses for the killifish, *Fundulus heteroclitus*. *Molecular ecology* **15**, 1109-1123.
- Almaça C (1995) Fish species and varieties introduced into Portuguese inland waters, p. 29, Museu do Bocage (Museu Nacional de História Natural), Lisbon, Portugal.
- Arias A, Drake P (1986) Contribución al conocimiento de la biología de *Valencia hispanica* Val., 1846 (Pisces: Ciprinodontidae), en el SO ibérico. *Inv. Pesq* **50**, 23-26.
- Avise JC (2004) Molecular markers, natural history and evolution, p. 668. Springer Science & Business Media, Massachusetts, USA.
- Baker-Dittus AM (1978) Foraging patterns of three sympatric killifish. *Copeia*, 383-389.
- Bax N, Williamson A, Aguero M, Gonzalez E, Geeves W (2003) Marine invasive alien species: a threat to global biodiversity. *Marine policy* **27**, 313-323.
- Bernardi G, Fernandez-Delgado C, Gomez-Chiarri M, Powers D (1995) Origin of a Spanish population of *Fundulus heteroclitus* inferred by cytochrome b sequence analysis. *Journal of Fish Biology* **47**, 737-740.
- Bernardi G, Sordino P, Powers DA (1993) Concordant mitochondrial and nuclear DNA phylogenies for populations of the teleost fish *Fundulus heteroclitus*. *Proceedings of the National Academy of Sciences* **90**, 9271-9274.
- Bianco P, Miller R (1989) First record of *Valencia letourneuxi* (Sauvage, 1880) in Peloponnese (Greece) and remarks on the Mediterranean family Valenciidae (Cyprinodontiformes). *Cybium* **13**, 385-387.
- Bigelow HB, Schroeder WC (1953) *Fishes of the Gulf of Maine*. US Government Printing Office, Washington, DC.  
[https://archive.org/stream/fishesofgulfofma1953bige/fishesofgulfofma1953bige\\_djvu.txt](https://archive.org/stream/fishesofgulfofma1953bige/fishesofgulfofma1953bige_djvu.txt)
- Blackburn TM, Pyšek P, Bacher S, *et al.* (2011) A proposed unified framework for biological invasions. *Trends in ecology & evolution* **26**, 333-339.
- Bock DG, Caseys C, Cousens RD, *et al.* (2015) What we still don't know about invasion genetics. *Molecular ecology* **24**, 2277-2297.
- Briski E, Bailey SA, Casas-Monroy O, *et al.* (2012) Relationship between propagule pressure and colonization pressure in invasion ecology: a test with ships' ballast. *Proceedings of the Royal Society of London B: Biological Sciences*, rspb20112671.
- Brook BW, Sodhi NS, Bradshaw CJ (2008) Synergies among extinction drivers under global change. *Trends in ecology & evolution* **23**, 453-460.
- Brown BL, Chapman RW (1991) Gene flow and mitochondrial DNA variation in the killifish, *Fundulus heteroclitus*. *Evolution* **45**, 1147-1161.
- Bulger AJ (1984) A daily rhythm in heat tolerance in the salt marsh fish *Fundulus heteroclitus*. *Journal of Experimental Zoology* **230**, 11-16.
- Burnett KG, Bain LJ, Baldwin WS, *et al.* (2007) *Fundulus* as the premier teleost model in environmental biology: opportunities for new insights using genomics. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* **2**, 257-286.

- Butner A, Brattstrom BH (1960) Local movement in *Menidia* and *Fundulus*. *Copeia* **1960**, 139-141.
- Carlton JT (2001a) *Introduced species in U.S. coastal waters: environmental impacts and management priorities*. Pew Oceans Commission, Arlington, VA, USA. [https://www.iatp.org/sites/default/files/Introduced\\_Species\\_in\\_US\\_Coastal\\_Waters\\_Enviro.htm](https://www.iatp.org/sites/default/files/Introduced_Species_in_US_Coastal_Waters_Enviro.htm)
- Carlton JT (2001b) The scale and ecological consequences of biological invasions in the World's oceans. In: *Invasive species and biodiversity management* (eds. Sandlund O, Schei P, Viken A), pp. 195-212, Dordrecht: Kluwer Academic Publishers.
- Carver C, Mallet A (2002) An assessment of the risk of ballast water-mediated introduction of non-indigenous phytoplankton and zooplankton into atlantic canadian waters. *Dartmouth, NS: Transport Canada*.
- Cashon RE, Van Beneden RJ, Powers DA (1981) Biochemical genetics of *Fundulus heteroclitus* (L.). IV. Spatial variation in gene frequencies of *Idh-A*, *Idh-B*, *6-Pgdh-A*, and *Est-S*. *Biochemical genetics* **19**, 715-728.
- Catry T, Ramos JA, Paiva VH, *et al.* (2006) Intercolony and annual differences in the diet and feeding ecology of little tern adults and chicks in Portugal. *The Condor* **108**, 366-376.
- Chapman JW, Miller TW, Coan EV (2003) Live seafood species as recipes for invasion. *Conservation Biology* **17**, 1386-1395.
- Chidester F (1920) The behavior of *Fundulus heteroclitus* on the salt marshes of New Jersey. *American Naturalist* **54**, 551-557.
- Christiansen FB, Frydenberg O (1974) Geographical patterns of four polymorphisms in *Zoarcetes viviparus* as evidence of selection. *Genetics* **77**, 765-770.
- Clavero M, Blanco-Garrido F, Prenda J (2007) Population and microhabitat effects of interspecific interactions on the endangered Andalusian toothcarp (*Aphanius baeticus*). *Environmental biology of fishes* **78**, 173-182.
- Coelho M, Gomes J, Re P (1976) *Valencia hispanica*, a new fish to Portugal. *Archivos do Museu Bocage (2a serie). Notas e suplementos*, 1-3.
- Connelly NA, O'Neill CR, Knuth BA, Brown TL (2007) Economic impacts of zebra mussels on drinking water treatment and electric power generation facilities. *Environmental Management* **40**, 105-112.
- Corbin KW (1977) Phosphoglucose isomerase polymorphism and natural selection in the sand crab, *Emerita talpoida*. *Evolution*, 331-340.
- Coutts AD, Dodgshun TJ (2007) The nature and extent of organisms in vessel sea-chests: a protected mechanism for marine bioinvasions. *Marine pollution bulletin* **54**, 875-886.
- Cristescu ME (2015) Genetic reconstructions of invasion history. *Molecular ecology* **24**, 2212-2225.
- Crivelli AJ (2006a) *Aphanius baeticus*. *The IUCN Red List of Threatened Species 2006: e.T61235A12452237*. <http://dx.doi.org/10.2305/IUCN.UK.2006.RLTS.T61235A12452237.en>
- Crivelli AJ (2006b) *Aphanius iberus*. *The IUCN Red List of Threatened Species 2006: e.T1846A8299534*. <https://doi.org/10.2305/IUCN.UK.2006.RLTS.T1846A8299534.en>
- Crivelli AJ (2006c) *Valencia hispanica*. *The IUCN Red List of Threatened Species 2006: e.T22829A9392487*. <https://doi.org/10.2305/IUCN.UK.2006.RLTS.T22829A9392487.en>
- Davidson IC, Brown CW, Sytsma MD, Ruiz GM (2009) The role of containerships as transfer mechanisms of marine biofouling species. *Biofouling* **25**, 645-655.
- Delibes M, Adrián I (1987) Effects of crayfish introduction on otter *Lutra lutra* food in the Doñana National Park, SW Spain. *Biological Conservation* **42**, 153-159.

- DiMichele L, Powers DA (1982) LDH-B genotype-specific hatching times of *Fundulus heteroclitus* embryos. *Nature* **296**, 563-564.
- Doadrio I, Carmona JA, Fernandez-Delgado C (2002) Morphometric study of the Iberian *Aphanius* (Actinopterygii, Cyprinodontiformes), with description of a new species. *Folia Zoologica* **51**, 67-80.
- Donnelly JP, Driscoll NW, Uchupi E, *et al.* (2005) Catastrophic meltwater discharge down the Hudson Valley: a potential trigger for the Intra-Allerød cold period. *Geology* **33**, 89-92.
- Duggan IC, Rixon CA, MacIsaac HJ (2006) Popularity and propagule pressure: determinants of introduction and establishment of aquarium fish. *Biological invasions* **8**, 377-382.
- Duvernell DD, Lindmeier JB, Faust KE, Whitehead A (2008) Relative influences of historical and contemporary forces shaping the distribution of genetic variation in the Atlantic killifish, *Fundulus heteroclitus*. *Molecular ecology* **17**, 1344-1360.
- Elvira B (1996) Endangered freshwater fish of Spain. In: *Conservation of endangered freshwater fish in Europe* (eds. Kirchhofer A, Hefti D), pp. 55-61. Birkhäuser Basel, Switzerland.
- Elvira B, Almodóvar A (2001) Freshwater fish introductions in Spain: facts and figures at the beginning of the 21st century. *Journal of Fish Biology* **59**, 323-331.
- Endler JA (1977) Geographic variation, speciation, and clines, p. 262. Princeton University Press.
- Estoup A, Guillemaud T (2010) Reconstructing routes of invasion using genetic data: why, how and so what? *Molecular ecology* **19**, 4113-4130.
- Fangue NA, Hofmeister M, Schulte PM (2006) Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*. *Journal of Experimental Biology* **209**, 2859-2872.
- Fernández-Delgado C (1989) Life-history patterns of the salt-marsh killifish *Fundulus heteroclitus* (L.) introduced in the estuary of the Guadalquivir River (South West Spain). *Estuarine, Coastal and Shelf Science* **29**, 573-582.
- Fernández-Delgado C (2010) Especies introducidas en Andalucía: El caso de los peces. *Especies exóticas invasoras en Andalucía. Talleres provinciales 2004-2006*, 132-137.
- Fernández-Delgado C, Hernando J, Herrera M, Bellido M (1986) Sobre el status taxonómico del género *Valencia* Myers, 1928 en el suroeste de Iberia. *Donana, Acta Vertebrata* **13**, 161-163.
- Fernández-Delgado C, Torralva MM, Oliva F, Pintos R (1999) Caracterización ecológica del hábitat del fartet (*Lebias ibera* Valenciennes, 1846) en una pequeña cuenca hidrográfica del bajo Guadalquivir. In: *Peces cyprinodontidos ibéricos: Fartet y Samaruc* (ed. Generalitat Valenciana), pp. 99-114. Consellería de Medio Ambiente, Valencia.
- Fernández-Pedrosa V, Latorre A, González A (1996) Evidence from mtDNA RFLP analysis for the introduction of *Fundulus heteroclitus* to southwestern Spain. *Journal of Fish Biology* **48**, 1278-1282.
- Franch N, Clavero M, Garrido M, *et al.* (2008) On the establishment and range expansion of oriental weatherfish (*Misgurnus anguillicaudatus*) in NE Iberian Peninsula. *Biological invasions* **10**, 1327-1331.
- Fritz E, Meredith W, Lotrich V (1975) Fall and winter movements and activity level of the mummichog, *Fundulus heteroclitus*, in a tidal creek. *Chesapeake Science* **16**, 211-215.
- Fuller P, Benson A, Maynard E, *et al.* (2017) *Neogobius melanostomus*. . USGS nonindigenous aquatic species database, Gainesville, FL. <https://nas.er.usgs.gov/queries/FactSheet.aspx?speciesID=713> Revision Date: 1/7/2016
- Galil B, Marchini A, Occhipinti-Ambrogi A, *et al.* (2014) International arrivals: widespread bioinvasions in European Seas. *Ethology Ecology & Evolution* **26**, 152-171.

- Galil BS, Marchini A, Occhipinti-Ambrogi A (2016) East is east and West is west? Management of marine bioinvasions in the Mediterranean Sea. *Estuarine, Coastal and Shelf Science*.
- García-Berthou E, Boix D, Clavero M (2007) Non-indigenous animal species naturalized in Iberian inland waters. In: *Biological invaders in inland waters: profiles, distribution, and threats. Invading Nature - Springer Series In Invasion Ecology* (ed. Gherardi F), pp. 123-140. Springer, Dordrecht.
- García-Llorente M, Martín-López B, González JA, Alcorlo P, Montes C (2008) Social perceptions of the impacts and benefits of invasive alien species: Implications for management. *Biological Conservation* **141**, 2969-2983.
- García-Revilla MG, Fernández-Delgado C (2009) *La introducción por mar de especies exóticas invasoras a través del agua de lastre de los barcos: el caso de Doñana*. Servicio de Publicaciones, Universidad de Córdoba. pp. 176. [www.uco.es/aphanius/includes/descargapub.php?id=8](http://www.uco.es/aphanius/includes/descargapub.php?id=8)
- Garside E, Chin-Yuen-Kee Z (1972) Influence of osmotic stress on upper lethal temperatures in the cyprinodontid fish *Fundulus heteroclitus* (L.). *Canadian Journal of Zoology* **50**, 787-791.
- Geller JB, Darling JA, Carlton JT (2010) Genetic perspectives on marine biological invasions. *Annual Review of Marine Science* **2**, 367-393.
- Ghabooli S, Shiganova TA, Briski E, et al. (2013) Invasion pathway of the ctenophore *Mnemiopsis leidyi* in the Mediterranean Sea. *PloS one* **8**, e81067.
- Gisbert E, López M (2007) First record of a population of the exotic mummichog *Fundulus heteroclitus* (L., 1766) in the Mediterranean Sea basin (Ebro River delta). *Journal of Fish Biology* **71**, 1220-1224.
- Gollasch S (2007) Is ballast water a major dispersal mechanism for marine organisms? In: *Biological invasions* (ed. Nentwig W), pp. 49-57. Springer-Verlag, Berlin.
- Gomez-Caruaana, Gomez P, Artal S (1984) Descripción de una nueva especie de pez continental ibérico, *Valencia lozanoi* n. sp.(Pisces, Cyprinodontidae). *Boletín de la Estación Central de Ecología*, 67-74.
- González-Vilaseñor LI, Powers DA (1990) Mitochondrial-DNA restriction-site polymorphisms in the teleost *Fundulus heteroclitus* support secondary intergradation. *Evolution* **44**, 27-37.
- Gozlan RE (2010) The cost of non-native aquatic species introductions in Spain: fact or fiction? *Aquatic Invasions* **5**, 231-238.
- Griffith RW (1974) Environment and salinity tolerance in the genus *Fundulus*. *Copeia* **1974**, 319-331.
- Grosholz E (2002) Ecological and evolutionary consequences of coastal invasions. *Trends in ecology & evolution* **17**, 22-27.
- Gutiérrez-Estrada J, Prenda J, Oliva F, Fernández-Delgado C (1998) Distribution and habitat preferences of the introduced mummichog *Fundulus heteroclitus* (Linnaeus) in south-western Spain. *Estuarine, Coastal and Shelf Science* **46**, 827-835.
- Handley L-JL, Estoup A, Evans D, et al. (2011) Ecological genetics of invasive alien species. *BioControl* **56**, 409.
- Haney RA, Dionne M, Puritz J, Rand DM (2009) The comparative phylogeography of east coast estuarine fishes in formerly glaciated sites: persistence versus recolonization in *Cyprinodon variegatus ovinus* and *Fundulus heteroclitus macrolepidotus*. *Journal of Heredity* **100**, 284-296.
- Hänfling B (2007) Understanding the establishment success of non-indigenous fishes: lessons from population genetics. *Journal of Fish Biology* **71**, 115-135.



- Hardy Jr JD (1978) *Development of fishes of the mid-Atlantic Bight*. Vol. II. *Anguillidae through Syngnathidae*. US Fish and Wildlife Service Publication FWS/OBS-78/12.
- Hernando J (1975) Nuevas localidades de *Valencia hispanica* (Pisces: Cyprinodontidae) en el suroeste de España. *Doñana Acta Vertebrata* **2**, 265-267.
- Hernando J, Soriguer M (1992) Biogeography of the freshwater fish of the Iberian Peninsula. *Limnetica* **8**, 243-253.
- Hulme PE (2009) Trade, transport and trouble: managing invasive species pathways in an era of globalization. *Journal of applied ecology* **46**, 10-18.
- Jeffries HP (1972) Fatty-acid ecology of a tidal marsh. *Limnology and Oceanography* **17**, 433-440.
- Katsanevakis S, Crocetta F (2014) Pathways of introduction of marine alien species in European waters and the Mediterranean—A possible undermined role of marine litter, 61-68.
- Kilian JV, Klauda RJ, Widman S, *et al.* (2012) An assessment of a bait industry and angler behavior as a vector of invasive species. *Biological invasions* **14**, 1469-1481.
- Kneib RT (1984) Patterns in the utilization of the intertidal salt marsh by larvae and juveniles of *Fundulus heteroclitus* (Linnaeus) and *Fundulus luciae* (Baird). *Journal of Experimental Marine Biology and Ecology* **83**, 41-51.
- Kneib RT (1986) The role of *Fundulus heteroclitus* in salt marsh trophic dynamics. *American Zoologist* **26**, 259-269.
- Kneib RT (1987) Predation risk and use of intertidal habitats by young fishes and shrimp. *Ecology* **68**, 379-386.
- Kneib RT (1997) The role of tidal marshes in the ecology of estuarine nekton. *Oceanography and Marine Biology - An Annual Review* **35**, 163-220.
- Kneib RT, Stiven AE (1978) Growth, reproduction, and feeding of *Fundulus heteroclitus* (L.) on a North Carolina salt marsh. *Journal of Experimental Marine Biology and Ecology* **31**, 121-140.
- Kulhanek SA, Ricciardi A, Leung B (2011) Is invasion history a useful tool for predicting the impacts of the world's worst aquatic invasive species? *Ecological applications* **21**, 189-202.
- Leunda PM (2010) Impacts of non-native fishes on Iberian freshwater ichthyofauna: current knowledge and gaps. *Aquatic Invasions* **5**, 239-262.
- Lotrich VA (1975) Summer home range and movements of *Fundulus heteroclitus* (Pisces: Cyprinodontidae) in a tidal creek. *Ecology* **56**, 191-198.
- Maceda-Veiga A (2013) Towards the conservation of freshwater fish: Iberian Rivers as an example of threats and management practices. *Reviews in Fish Biology and Fisheries* **23**, 1-22.
- Machado A (1857) Catálogo de los peces que habitan o frecuentan las costas de Cádiz y Huelva, con inclusión de los del Rio Guadalquivir, p. 43. Imprenta Española y Extranjera, Sevilla.
- McKenzie JL, Dhillon RS, Schulte PM (2015) Evidence for a bimodal distribution of hybrid indices in a hybrid zone with high admixture. *Royal Society open science* **2**, 150285.
- McKenzie JL, Dhillon RS, Schulte PM (2016) Steep, coincident, and concordant clines in mitochondrial and nuclear-encoded genes in a hybrid zone between subspecies of Atlantic killifish, *Fundulus heteroclitus*. *Ecology and Evolution* **6**, 5771-5787.
- Millennium Ecosystem Assessment (2005) *Ecosystems and Human Well-being: Biodiversity Synthesis*. World Resources Institute, Washington, DC. <http://millenniumassessment.org/en/Synthesis.html>
- Mineur F, Johnson MP, Maggs CA (2008) Macroalgal introductions by hull fouling on recreational vessels: seaweeds and sailors. *Environmental Management* **42**, 667-676.

- Molnar JL, Gamboa RL, Revenga C, Spalding MD (2008) Assessing the global threat of invasive species to marine biodiversity. *Frontiers in Ecology and the Environment* **6**, 485-492.
- Morin RP, Able KW (1983) Patterns of geographic variation in the egg morphology of the fundulid fish, *Fundulus heteroclitus*. *Copeia* **1983**, 726-740.
- Naylor RL, Williams SL, Strong DR (2001) Aquaculture—A gateway for exotic species. *Science* **294**, 1655-1656.
- Nevo E (1976) Adaptive strategies of genetic systems in constant and varying environments. In: *Population Genetics and Ecology* (eds. Karlin S, Nevo E), pp. 141-158. Academic Press, New York.
- North American Native Fishes Association (2010) *Fundulus heteroclitus* (*Mummichog*). <https://goo.gl/sNRCQx>
- Olenin S, Narščius A, Minchin D, *et al.* (2014) Making non-indigenous species information systems practical for management and useful for research: An aquatic perspective. *Biological Conservation* **173**, 98-107.
- Oliva-Paterna FJ, Doadrio I, Fernández-Delgado C (2006) Threatened fishes of the world: *Aphanius baeticus* (Doadrio, Carmona & Fernández-Delgado, 2002)(Cyprinodontidae). *Environmental biology of fishes* **75**, 415-417.
- Otero M, Cebrian E, Francour P, Galil B, Savini D (2013) Monitoring Marine Invasive Species in Mediterranean Marine Protected Areas (MPAs): A strategy and practical guide for managers. *Malaga, Spain: IUCN*, 136.
- Padilla DK, Williams SL (2004) Beyond ballast water: aquarium and ornamental trades as sources of invasive species in aquatic ecosystems. *Frontiers in Ecology and the Environment* **2**, 131-138.
- Paiva VH, Ramos JA, Machado D, *et al.* (2006) Importance of marine prey to growth of estuarine tern chicks: evidence from an energetic balance model, 241-255.
- Place A, Powers D (1977) Liver LDH allozymes of *Fundulus heteroclitus*: A selective adaptation or genetic drift **36**, 738-738.
- Place AR, Powers DA (1979) Genetic variation and relative catalytic efficiencies: lactate dehydrogenase B allozymes of *Fundulus heteroclitus*. *Proceedings of the National Academy of Sciences* **76**, 2354-2358.
- Planelles M, Reyna S (1996) Conservation of samaruc, *Valencia hispanica* (Valenciennes, 1846),(Pisces: Cyprinodontidae), an endemic and endangered species, in the community of Valencia (east Spain). In: *Conservation of Endangered Freshwater Fish in Europe* (eds. Kirchhofer A, Hefti D), pp. 329-335. Birkhäuser Basel, Switzerland.
- Powers D (1972) Enzyme kinetics in predicting gene frequencies of natural populations. *Journal of the American Society of Ichthyologists and Herpetologists* **52**, 77.
- Powers DA, Lauerman T, Crawford D, DiMichele L (1991) Genetic mechanisms for adapting to a changing environment. *Annual review of genetics* **25**, 629-660.
- Powers DA, Place AR (1978) Biochemical genetics of *Fundulus heteroclitus* (L.). I. Temporal and spatial variation in gene frequencies of *Ldh-B*, *Mdh-A*, *Gpi-B*, and *Pgm-A*. *Biochemical genetics* **16**, 593-607.
- Powers DA, Powers D (1975) Predicting gene frequencies in natural populations: a testable hypothesis. In: *The isozymes, IV Genetics and evolution* (ed. Markert C), pp. 63-84. Academic Press, New York.
- Powers DA, Ropson I, Brown DC, *et al.* (1986) Genetic variation in *Fundulus heteroclitus*: geographic distribution. *American Zoologist* **26**, 131-144.
- Powers DA, Schulte PM (1998) Evolutionary adaptations of gene structure and expression in natural populations in relation to a changing environment: a multidisciplinary approach

- to address the million-year saga of a small fish. *The Journal of experimental zoology* **282**, 71-94.
- Prinslow TE, Valiela I, Teal JM (1974) The effect of detritus and ration size on the growth of *Fundulus heteroclitus* (L.). *Journal of Experimental Marine Biology and Ecology* **16**, 1-10.
- Rhyne AL, Tlusty MF, Schofield PJ, *et al.* (2012) Revealing the appetite of the marine aquarium fish trade: the volume and biodiversity of fish imported into the United States. *PloS one* **7**, e35808.
- Ribeiro F, Elvira B, Collares-Pereira MJ, Moyle PB (2008) Life-history traits of non-native fishes in Iberian watersheds across several invasion stages: a first approach. *Biological invasions* **10**, 89-102.
- Rius M, Turon X, Bernardi G, Volckaert FA, Viard F (2014) Marine invasion genetics: from spatio-temporal patterns to evolutionary outcomes. *Biological invasions* **17**, 869-885.
- Roderick GK, Navajas M (2003) Genes in new environments: genetics and evolution in biological control. *Nature Reviews Genetics* **4**, 889-899.
- Ropson IJ, Brown DC, Powers DA (1990) Biochemical genetics of *Fundulus heteroclitus* (L.). VI. Geographical variation in the gene frequencies of 15 loci. *Evolution* **44**, 16-26.
- Rosenfield JA, Nolasco S, Lindauer S, Sandoval C, Kodric-Brown A (2004) The role of hybrid vigor in the replacement of Pecos pupfish by its hybrids with sheepshead minnow. *Conservation Biology* **18**, 1589-1598.
- Ruiz GM, Carlton JT, Grosholz ED, Hines AH (1997) Global invasions of marine and estuarine habitats by non-indigenous species: mechanisms, extent, and consequences. *American Zoologist* **37**, 621-632.
- Schulte PM (2001) Environmental adaptations as windows on molecular evolution. *Comparative biochemistry and physiology part B: Biochemistry and Molecular Biology* **128**, 597-611.
- Scott GR, Rogers JT, Richards JG, Wood CM, Schulte PM (2004) Intraspecific divergence of ionoregulatory physiology in the euryhaline teleost *Fundulus heteroclitus*: possible mechanisms of freshwater adaptation. *Journal of Experimental Biology* **207**, 3399-3410.
- Shiganova T (1998) Invasion of the Black Sea by the ctenophore *Mnemiopsis leidyi* and recent changes in pelagic community structure. *Fisheries Oceanography* **7**, 305-310.
- Simberloff D, Von Holle B (1999) Positive interactions of nonindigenous species: invasional meltdown? *Biological invasions* **1**, 21-32.
- Skinner MA, Courtenay SC, Parker WR, Curry RA (2005) Site Fidelity of Mummichogs (*Fundulus heteroclitus*) in an Atlantic Canadian Estuary. *Water Quality Research Journal of Canada* **40**, 288-298.
- Smith KJ, Taghon GL, Able KW (2002) Trophic linkages in marshes: ontogenetic changes in diet for young-of-the-year mummichog, *Fundulus heteroclitus*. In: *Concepts and controversies in tidal marsh ecology* (eds. Weinstein MP, Kreeger DA), pp. 222-237. Springer, Dordrecht.
- Smith MW, Chapman RW, Powers DA (1998) Mitochondrial DNA analysis of Atlantic coast, Chesapeake Bay, and Delaware Bay populations of the teleost *Fundulus heteroclitus* indicates temporally unstable distributions over geologic time. *Molecular Marine Biology and Biotechnology* **7**, 79-87.
- Stepien CA, Brown JE, Neilson ME, Tumeo MA (2005) Genetic diversity of invasive species in the Great Lakes versus their Eurasian source populations: insights for risk analysis. *Risk Analysis* **25**, 1043-1060.

- Strand AE, Williams LM, Oleksiak MF, Sotka EE (2012) Can diversifying selection be distinguished from history in geographic clines? A population genomic study of killifish (*Fundulus heteroclitus*). *PloS one* **7**, e45138.
- Strayer DL (2009) Twenty years of zebra mussels: lessons from the mollusk that made headlines. *Frontiers in Ecology and the Environment* **7**, 135-141.
- Sweeney J, Deegan L, Garritt R (1998) Population size and site fidelity of *Fundulus heteroclitus* in a macrotidal saltmarsh creek. *The Biological Bulletin* **195**, 238.
- Taylor MH, Di Michele L, Leach GJ (1977) Egg stranding in the life cycle of the mummichog, *Fundulus heteroclitus*. *Copeia* **1977**, 397-399.
- Taylor MH, DiMichele L (1983) Spawning site utilization in a Delaware population of *Fundulus heteroclitus* (Pisces: Cyprinodontidae). *Copeia* **1983**, 719-725.
- Taylor MH, Leach GJ, DiMichele L, Levitan WM, Jacob WF (1979) Lunar spawning cycle in the mummichog, *Fundulus heteroclitus* (Pisces: Cyprinodontidae). *Copeia* **1979**, 291-297.
- Teo S, Able K (2003) Habitat use and movement of the mummichog (*Fundulus heteroclitus*) in a restored salt marsh. *Estuaries* **26**, 720-730.
- Umminger BL (1972) Physiological studies on supercooled killifish (*Fundulus heteroclitus*). IV. Carbohydrate metabolism in hypophysectomized killifish at subzero temperatures. *Journal of Experimental Zoology* **181**, 217-222.
- Valiela I, Wright J, Teal JM, Volkmann S (1977) Growth, production and energy transformations in the salt-marsh killifish *Fundulus heteroclitus*. *Marine Biology* **40**, 135-144.
- Van Beneden RJ, Cashon RE, Powers DA (1981) Biochemical genetics of *Fundulus heteroclitus* (L.). III. Inheritance of isocitrate dehydrogenase (*Idh-A* and *Idh-B*), 6-phosphogluconate dehydrogenase (*6-Pgdh-A*), and serum esterase (*Est-S*) polymorphisms. *Biochemical genetics* **19**, 701-714.
- Vilà M, Basnou C, Pyšek P, *et al.* (2010) How well do we understand the impacts of alien species on ecosystem services? A pan-European, cross-taxa assessment. *Frontiers in Ecology and the Environment* **8**, 135-144.
- Vince S, Valiela I, Backus N, Teal J (1976) Predation by the salt marsh killifish *Fundulus heteroclitus* (L.) in relation to prey size and habitat structure: consequences for prey distribution and abundance. *Journal of Experimental Marine Biology and Ecology* **23**, 255-266.
- Wallentinus I, Nyberg CD (2007) Introduced marine organisms as habitat modifiers. *Marine pollution bulletin* **55**, 323-332.
- Wanless RM, Scott S, Sauer WH, *et al.* (2010) Semi-submersible rigs: a vector transporting entire marine communities around the world. *Biological invasions* **12**, 2573-2583.
- Weisberg S, Lotrich V (1982) The importance of an infrequently flooded intertidal marsh surface as an energy source for the mummichog *Fundulus heteroclitus*: an experimental approach. *Marine Biology* **66**, 307-310.
- Weisberg SB, Whalen R, Lotrich V (1981) Tidal and diurnal influence on food consumption of a salt marsh killifish *Fundulus heteroclitus*. *Marine Biology* **61**, 243-246.
- Whitehead A (2009) Comparative mitochondrial genomics within and among species of killifish. *BMC Evolutionary Biology* **9**, 11.
- Whitt GS (1969) Homology of lactate dehydrogenase genes: E gene function in the teleost nervous system. *Science* **166**, 1156-1158.
- Williams LM, Ma X, Boyko AR, Bustamante CD, Oleksiak MF (2010) SNP identification, verification, and utility for population genetics in a non-model genus. *BMC genetics* **11**, 1.

- Williams SL, Davidson IC, Pasari JR, *et al.* (2013) Managing multiple vectors for marine invasions in an increasingly connected world. *Bioscience* **63**, 952-966.
- Zenetos A, Gofas S, Morri C, *et al.* (2012) Alien species in the Mediterranean Sea by 2012. A contribution to the application of European Union's Marine Strategy Framework Directive (MSFD). Part 2. Introduction trends and pathways. *Mediterranean Marine Science* **13**, 328-352.

## **CHAPTER 2: INVASIVE GENETICS OF THE MUMMICHOG (*FUNDULUS HETEROCLITUS*): recent anthropogenic introduction in Iberia**

Morim<sup>1</sup>, Teófilo, Madeira<sup>1</sup>, Pedro M., Palma<sup>1</sup>, J., Duvernell<sup>2</sup>, David D., Gisbert<sup>3</sup>, Enric., Cunha<sup>1</sup>, Regina L. and Castilho\*<sup>1</sup>, Rita

<sup>1</sup>Centre for Marine Sciences, CCMAR-CIMAR Laboratório Associado, Campus de Gambelas, 8005-139 Faro, Portugal

<sup>2</sup>Department of Biological Sciences, Southern Illinois University Edwardsville, Edwardsville, Illinois 62026, USA

<sup>3</sup>RTA - Sant Carles de la Ràpita, Crta Poble Nou km 5.5, P.O. Box 200, 43540 Sant Carles de la Ràpita, SPAIN

\*Corresponding author:

Centro de Ciências do Mar (CCMAR), Campus de Gambelas, 8005-139 Faro, Portugal. Tel: +351 918397282, Fax: +351 289 800 069.

Keywords: introduction vector, cytochrome *b*, mitochondrial DNA, invasive species

### **Abstract**

Human activities such as trade and transport have increased considerably in the last decades, greatly facilitating the introduction and spread of non-native species at a global level. In the Iberian Peninsula, *Fundulus heteroclitus*, a small euryhaline coastal fish with short dispersal, was found for the first time in the mid-1970s. Since then, *F. heteroclitus* underwent range expansions, colonizing the southern region of Portugal, southwestern coast of Spain and the Ebro Delta in the Mediterranean Sea. We analysed mitochondrial DNA cytochrome *b* sequences to elucidate the invasion pathways in Iberia. We sampled three Iberian locations (Faro, Cádiz and Ebro Delta) and 13 other locations along *F. heteroclitus* native range in North America. Results revealed a single haplotype, common to all invasive populations, which can be traced to the northern region of the species native range. Thus, we suggest the origin of the founder individuals to be between New York and Nova Scotia. Additionally, the lack of genetic structure within Iberia is consistent with a recent invasion scenario and a strong founder effect. We suggest the most probable introduction vector is associated to aquarium trade and we further

discuss the hypothesis of a second human mediated introduction responsible for the establishment of individuals in the Ebro Delta. Given the high tolerance to salinity and temperature, the species will most likely continue to spread geographically, as long as habitat requirements remain available.

## **Introduction**

As a consequence of human activities involving large distance marine transportation and trade, worldwide marine biological invasion rates have increased dramatically in the last 30 years (Bax *et al.* 2003; Hulme 2009; Meyerson & Mooney 2007; Perrings *et al.* 2005). In the European seas there are over 850 invasive species, of which 237 along the western European margins, 680 in the Mediterranean Sea and the remaining in the Baltic (Galil *et al.* 2014). Marine invasive species pose a significant environmental threat as they are one of the major drivers of biodiversity loss (Millennium Ecosystem Assessment 2005), altering ecosystems and their dynamics, shifting the community structure and displacing endemic species (Bax *et al.* 2003; Molnar *et al.* 2008; Wallentinus & Nyberg 2007). Negative impacts may also be registered at the economic and social levels, affecting fisheries, aquacultures, tourism or human health (Bax *et al.* 2003; Molnar *et al.* 2008). Invasive species spread and occupy new marine and coastal ecosystems through several maritime introduction vectors such as ballast water, biofouling of vessels, aquaculture escape or ornamental species trade (see Williams *et al.* 2013 and references therein).

After becoming established, it is almost impossible to completely eradicate invasive non-native species (Katsanevakis & Crocetta 2014). Thereby, preventing the establishment of new invasive species is of extreme importance and probably the best way to avoid the negative impacts associated to the spread of non-native species (Katsanevakis & Crocetta 2014; Otero *et al.* 2013). Furthermore, a deep understanding of the invasion pathways and population sources is relevant not only to prevent new invasions, but also to aid the development of effective conservation measures (Galil *et al.* 2014; Ghabooli *et al.* 2013; Handley *et al.* 2011). For instance, information on the ecology of the invasive species within its native range may help choosing adequate biological control agents for containment measures (Roderick & Navajas 2003). Knowledge on the introduction vectors, population sources and their genetic characteristics can be used to create risk maps, which provide useful insights into the potential ecological impacts (Kulhanek *et al.* 2011), predicting the impacts of different routes and the probability of occurring new invasions (Hulme 2009; Molnar *et al.* 2008).

However, reconstructing the invasion pathways and identifying the putative source populations with historical and contemporary vector records is a difficult task (Estoup & Guillemaud 2010; Handley *et al.* 2011). In this context, molecular genetic data is a powerful tool to reconstruct the invasive history by identifying putative source populations and genetic bottlenecks (Bock *et al.* 2015; Cristescu 2015; Estoup & Guillemaud 2010; Handley *et al.* 2011). Although genetic data does not always allow for successful identification of these processes, there are three conditions which increase the probability of accurate reconstruction of the invasive pathway, all fulfilled for *Fundulus heteroclitus*: extensive sampling along the native range to ensure all the putative source populations are known, the presence of genetic structure in the native range to narrow down the putative source regions and a short amount of time passed since the invasion so that processes such as genetic drift do not increase the genetic differentiation between native and invasive populations (Geller *et al.* 2010).

The mummichog, *Fundulus heteroclitus* (Linnaeus, 1766), is a small teleost naturally occurring almost continuously in saltmarshes of the North American east coast, from Newfoundland to Florida (Hardy Jr 1978). This species is extremely resistant to a wide range of salinities and temperatures, and can be found in freshwater, brackish or saltwater, inhabiting sheltered coastal areas such as saltmarshes, tidal creeks, estuaries or bays all year-round (Bigelow & Schroeder 1953; Hardy Jr 1978). *Fundulus heteroclitus* is one of the most stationary marine species (Bigelow & Schroeder 1953), with short dispersal distances (1-2 km, Fritz *et al.* 1975; Lotrich 1975), high site fidelity closely related to the saltmarshes (Kneib 1984) and short home ranges (36-38 m, Lotrich 1975).

*Fundulus heteroclitus* is an important model for the study of adaptive genetic clines, exhibiting distinct and extensive latitudinal clinal variation in relation to morphological, physiological and genetic traits along the North American Atlantic coast, which have been thoroughly studied for a long time (e.g., Powers *et al.* 1986). Geographical analysis of morphological traits (Able & Felley 1986; Morin & Able 1983), enzyme-coding loci (Powers & Place 1978; Powers *et al.* 1986; Ropson *et al.* 1990), mitochondrial DNA (Bernardi *et al.* 1993; González-Vilaseñor & Powers 1990; Smith *et al.* 1998), putative neutral nuclear microsatellite loci (Adams *et al.* 2006; Duvernell *et al.* 2008), and a variety of nuclear single-nucleotide polymorphisms (McKenzie *et al.* 2015, 2016; Strand *et al.* 2012; Williams *et al.* 2010) demonstrated concordance of clinal patterns, which break is placed between the meridians 40° and 41° N, along the coast of New Jersey. These studies concur with Morin & Able (1983), supporting the division into two subspecies: *F. heteroclitus heteroclitus*



(Linnaeus, 1766) at north of the cline and *F. heteroclitus macrolepidotus* (Walbaum, 1792) at south of the cline.

In the Iberian Peninsula, *F. heteroclitus* was first detected in the 1970s, in the Guadalquivir and Guadiana saltmarshes from the southwestern coast of Spain (Hernando 1975) and in the Guadiana Delta (Coelho *et al.* 1976). More recently, its presence was also reported in the Ebro Delta, northeastern coast of Spain (Gisbert & López 2007) and in the Ria Formosa, south of Portugal (e.g., Catry *et al.* 2006; Paiva *et al.* 2006b). The species was probably introduced between 1970 and 1973 in the Spanish saltmarshes (Fernández-Delgado 1989) either involuntarily via aquarium trade (Bernardi *et al.* 1995; Elvira & Almodóvar 2001; Hernando & Soriguer 1992), ballast water (Fernández-Delgado 2010; García-Revilla & Fernández-Delgado 2009), or intentionally for purposes of biological control (Gozlan 2010). The Ebro Delta individuals were probably caught in the Spanish saltmarshes to be used in aquaculture and aquarium trade (Gisbert & López 2007) and were later released from captivity. Another possibility for the Ebro Delta introduction is an accidental escape from a research centre nearby, where this species had already been used as a scientific model (Gisbert & López 2007). Two previous studies have analysed the origin of the southwestern Spanish populations using mitochondrial DNA (mtDNA), concluding the founder individuals were original from the northern native region between Maine and Nova Scotia where the northern subspecies *F. h. macrolepidotus* is distributed (Bernardi *et al.* 1995; Fernández-Pedrosa *et al.* 1996).

In the present study, we aim to build on previously published studies on the invasive range of *Fundulus heteroclitus* (Bernardi *et al.* 1995; Fernández-Pedrosa *et al.* 1996) by using (1) more sampling locations (one in the Mediterranean Sea and two locations in the eastern Atlantic, and thirteen native locations), (2) a significantly larger number of individuals (248 in total), and (3) a three times larger fragment of the mitochondrial DNA cytochrome *b* gene; and to evaluate the genetic diversity and invasion pathways. Given that the species was reported in the Iberian Peninsula very recently and has a limited adult dispersal capability, we tested the hypothesis of a human-mediated single Iberian introduction followed by dispersal promoted along the main oceanographic currents. This hypothesis leads to the expectation of an Iberian invasion based on a few founder individuals, with consequent lower genetic diversity than the putative identified source population. Also, the Iberian populations are expected to show no evident genetic structure given the short time since invasion.

## Materials and methods

**Sampling.** Samples from a total of 248 *F. heteroclitus* individuals were obtained from 16 locations: 13 sites in the western Atlantic, one in the Mediterranean Sea and two locations in the eastern Atlantic (Table 2.1 and Figure 2.1), and were stored in 96% ethanol and kept at -20 °C. The populations from the western Atlantic sampled above 40° N are hereafter referred to as northern locations (ID 1-8), while the others sampled below are referred to as southern locations (ID 9-13). The locations from the Mediterranean and eastern Atlantic are referred to as Iberian (ID 14-16).

Table 2.1. Sample location, sample abbreviations and summary statistics for a cytochrome *b* sequence fragment from *Fundulus heteroclitus*. ID refers to numbers in Figure 2.1.

Location	ID	Code	Latitude / longitude	<i>n</i>	<i>n<sub>h</sub></i>	<i>n<sub>p</sub></i>
Bridgewater	1	HV	44°22.0'N / 64°31.0'W	15	2	0
Chewonki	2	CM	43°57.3'N / 69°43.2'W	15	3	1
Wells	3	WM	43°19.2'N / 70°34.2'W	15	4	2
Woods Hole	4	WH	41°31.5'N / 70°40.4'W	16	9	4
Jerusalem	5	JR	41°23.1'N / 71°31.5'W	15	5	4
Clinton	6	CC	41°15.3'N / 72°32.8'W	16	7	6
Newark Bay	7	NB	40°41.2'N / 74°06.7'W	15	8	6
Red Bank	8	RE	40°20.9'N / 74°05.0'W	15	7	5
Tuckerton	9	TN	39°32.2'N / 74°19.4'W	15	10	8
Speace	10	SP	38°09.1'N / 75°17.2'W	15	9	6
Suffolk	11	CH	36°51.8'N / 76°28.7'W	16	7	3
Roanoke Island	12	RI	35°53.8'N / 75°36.9'W	15	9	7
Skidaway Island	13	SI	31°56.8'N / 81°04.2'W	16	11	10
Faro	14	RF	37°00.3'N / 07°58.0'W	16	1	0
Cádiz	15	CD	36°31.4'N / 06°11.4'W	17	1	0
Ebro Delta	16	ED	40°37.38'N / 0°39.44'E	16	1	0

*n*, number of individuals; *n<sub>h</sub>*, number of haplotypes; *n<sub>p</sub>*, number of private haplotypes

**DNA extraction, PCR amplification and sequencing.** Total genomic DNA was extracted from caudal fin tissue following a standard Chelex 100 protocol (Walsh *et al.* 1991). Extraction results were checked by electrophoresis in 0.8% agarose gel stained with GelRed. Polymerase Chain Reactions (PCR) were conducted in a total volume of 25 µL, with 1X buffer, 10 mM dNTPs, 10mM of each primer, 1U Taq Advantage 2 Polymerase mix DNA polymerase (CLONTECH-TaKaRa), 2 µL of DNA and Milli-Q water to the final volume. A fragment of the cytochrome *b* (*cyt b*) gene (1000 base pairs) was amplified with the forward primer

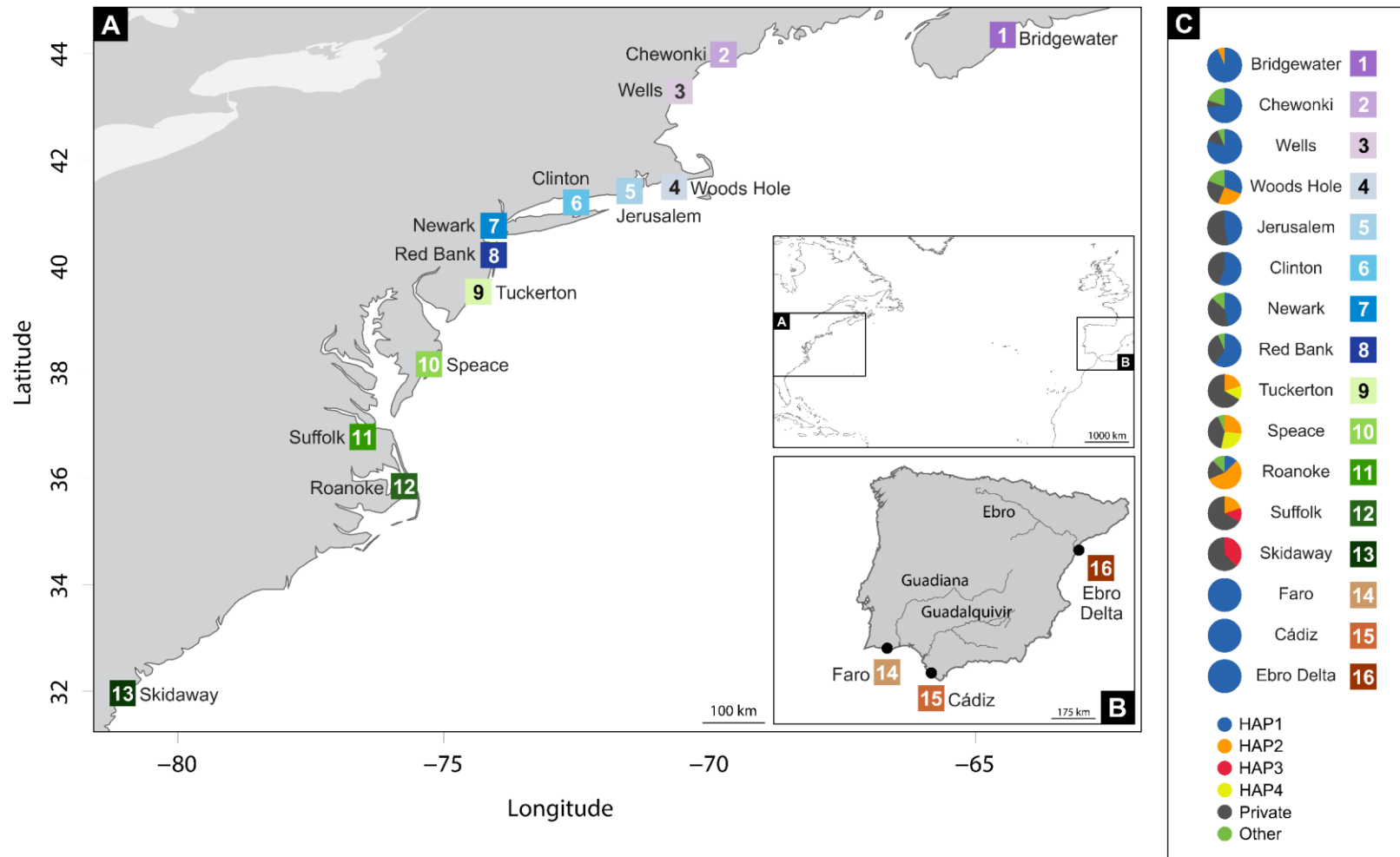


Figure 2.1. Distribution map showing the collection localities along the coast of North America (A), and Iberian Peninsula (B). Locations and sample details can be found in Table 2.1. Coloured circles display the distribution of the relative proportions of the *cyt b* haplotypes from each location: the four most common haplotypes, the private haplotypes, and all the other shared, but less frequent haplotypes, are represented (C, see legend).

GludG-L14724 (5'-TGACTTGAARAACCAAYCGTTG-3') (Palumbi *et al.* 1991) and the reverse primer cb6b.h (5'-GGAATTCACCTCTCCGGTTTACAAGAC-3') (Martin & Bermingham 1998). PCR amplification consists of an initial 4 min denaturation step at 95 °C, followed by 40 cycles of 1 min at 94 °C (denaturation), 1 min at 50 °C (annealing) and 1.5 min at 72 °C (extension), and a 5 min final extension step. When amplification was not successful the following profile was used: initial 3 min denaturation step at 95 °C, followed by 32 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 54 °C and extension for 1 min at 68 °C, and a final extension step for 4 min at 68 °C. PCR products were checked afterwards by electrophoresis in a 1% agarose gel stained with GelRed. Mitochondrial DNA was purified by ethanol/sodium acetate precipitation (Sambrook & Russel 2001). Its purity and quantity were analysed using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, USA). Sequencing was performed on an ABI 3130xl capillary sequencer (Applied Biosystems – CCMAR, Portugal) using the forward primer from the PCR amplification (GludG-L14724).

**Genetic analysis.** Cyt *b* sequences were aligned and manually checked using the software Geneious v4.8.2 (Biomatters, Ltd., Auckland, New Zealand). The number of haplotypes ( $n$ ), number of private haplotypes ( $n_p$ ), and the haplotype ( $h$ ) (Nei & Tajima 1981) and nucleotide diversities ( $\pi$ ) (Nei 1987) were calculated for each location using the DNAsP v5.10.1 (Librado & Rozas 2009). To represent the phylogeographic relationships among haplotypes, a haplotype network was constructed using the Median Joining algorithm implemented in NETWORK v5.0 (Bandelt *et al.* 1999, fluxus-engineering.com).

We used two approaches to infer the most probable source area of the introduced Iberian populations within the native range of *F. heteroclitus*: (1) the geographical distribution of haplotypes in native populations, and (2) the phylogeographical relationships among haplotypes.

## Results

A total of 248 specimens from 16 locations were analysed, resulting in cyt *b* sequences with a final length of 700 base pairs (bp), which comprised 77 (11%) polymorphic sites and 32 (41.6%) parsimony-informative sites. These polymorphisms defined 70 haplotypes, of which 62 (88.6%) are private haplotypes (present in one location only) and 55 (78.6%) are singletons (present in one individual only). Overall haplotype diversity was high ( $0.74 \pm 0.03$ ) ranging from null in Iberia (ID 14-16) to 0.93 in Tuckerton (ID 9) and Roanoke Island (ID 12), whereas

mean nucleotide diversity was low ( $0.26\% \pm 0.06\%$ ) ranging from null diversity in Iberia to 0.91% in Woods Hole (ID 4) (Table 2.1 and Figure 2.2).

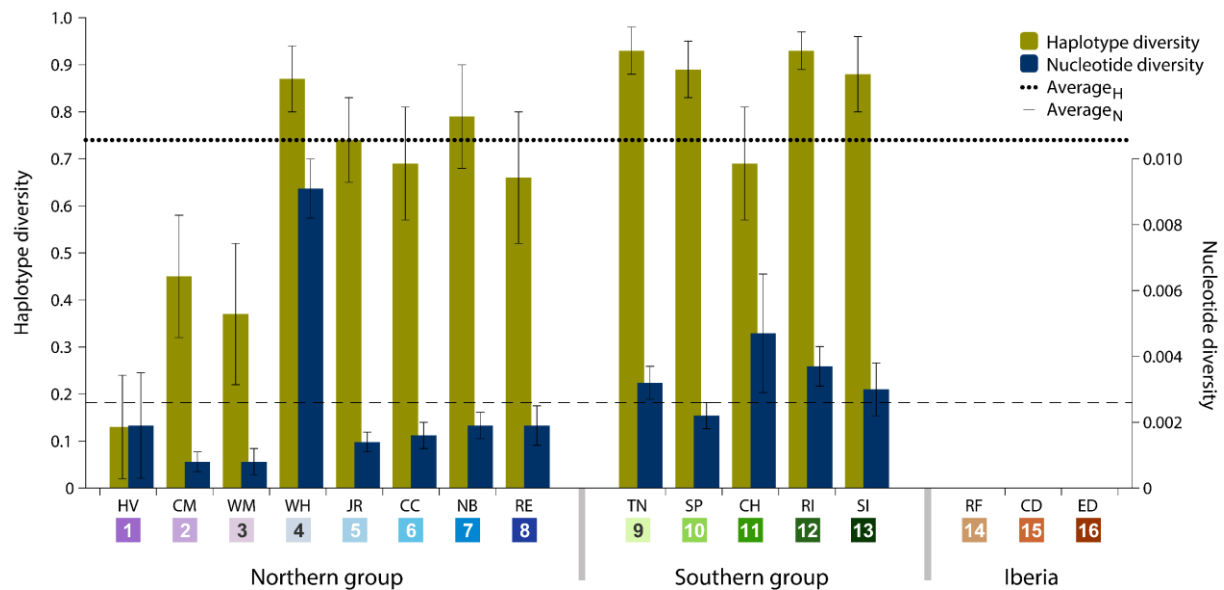


Figure 2.2. Haplotype and nucleotide diversity from all sampled locations. Abbreviations in the legend are defined in Table 2.1 and colours are the same as in Figure 2.1.

The most abundant haplotype in USA locations is shared by 50.4% ( $n = 125$ ) of the individuals and is present in all northern group locations, in one southern location and in all Iberian locations (Figure 2.1.C). This is the only haplotype detected in the invasive range of the species (Faro, Cádiz and in the Ebro Delta). The second most frequent haplotype in USA locations is shared by 9.7% ( $n = 24$ ) individuals in six locations, although in higher frequency in the southern group locations. Two other haplotypes were found in 3.2% ( $n = 8$ ) and in 2.4% ( $n = 6$ ) of the individuals from two locations. All other haplotypes were present in five or less individuals and in less than three locations (Figure 2.1.C).

The *cyt b* based haplotype network (Figure 2.3) displays two haplogroups separated by 9 mutational steps. One haplogroup is constituted by all eight northern group locations (Bridgewater to Red Bank, ID 1-8), two individuals from the southern group (Suffolk, ID 11) and by the Iberian locations (Faro to Ebro Delta, ID 14-16). The other haplogroup is formed by individuals from all southern locations (Tuckerton to Skidaway Island, ID 9-13) and includes eight individuals from northern locations: one from Bridgewater (ID 1) and seven from Woods Hole (ID 4). Overall, both haplogroups display star-like configurations with different levels of complexity. The northern haplogroup is simpler with 90% of the haplotypes separated by a single mutation, while the southern haplogroup is more complex, with 3 mini-stars

interconnected by a one mutation each, and haplotypes separated by up to five mutations. The haplotype found in the Iberia Peninsula belongs to the northern haplogroup.

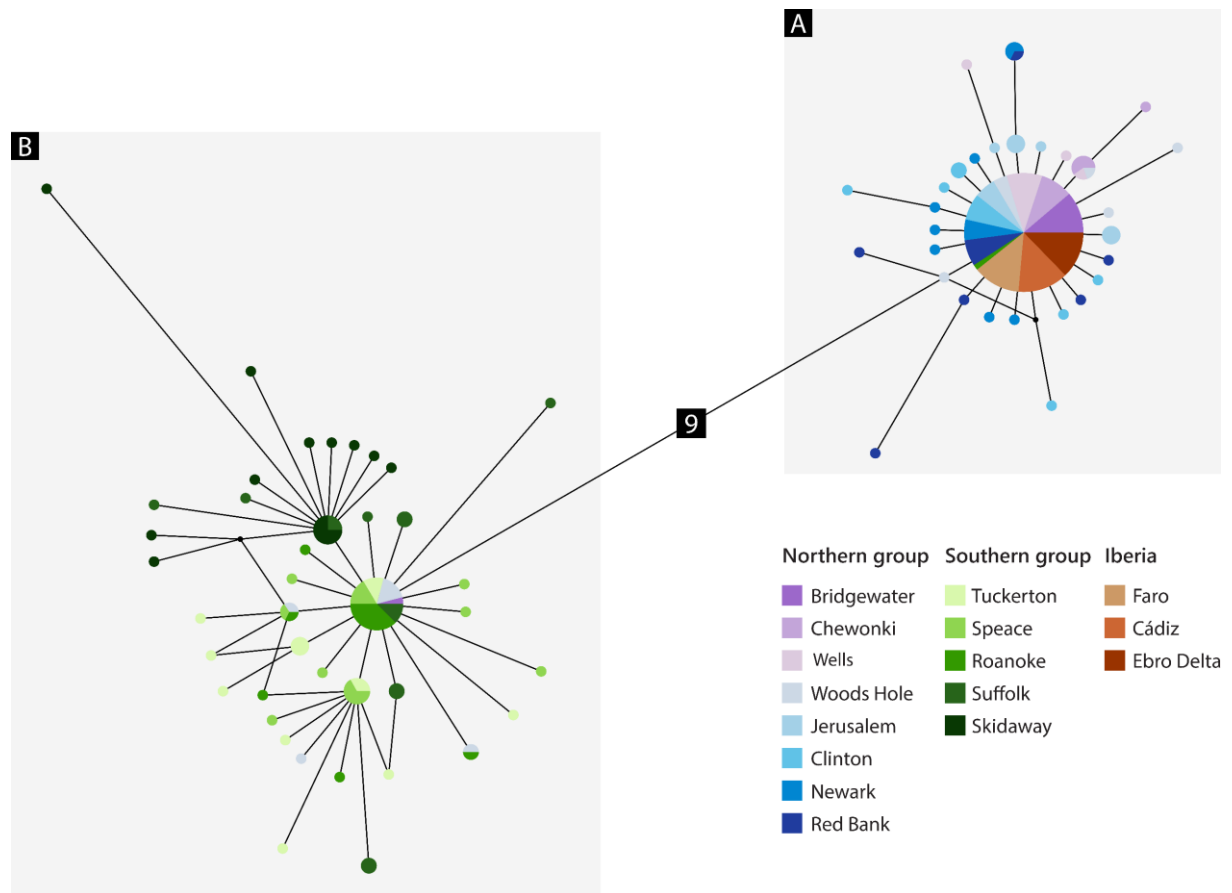


Figure 2.3. Median-joining cytochrome *b* haplotype network for *Fundulus heteroclitus*. Each circle represents a different haplotype with size proportional to the frequency of the haplotype within the sample. Line length is proportional to the number of mutations between haplotypes. Each colour corresponds to a different location and are the same as in Figure 2.1. Locations details can be found in Table 2.1. Black dots represent putative unsampled haplotypes and the number in black square shows the number of mutations separating haplogroup A (A) from the haplogroup B (B).

## Discussion

The *cyt b* sequences from the *Fundulus heteroclitus* invasive populations in Iberia revealed the presence of a single haplotype common to all individuals. This haplotype is the predominant haplotype in the northern group of the native distribution, and although we cannot determine which exact location was at the origin of the introduced individuals, one can identify the northern group as the source of the introduction. The lack of genetic diversity is consistent with a strong founder effect at the origin of *F. heteroclitus* in Iberia. Although there is no direct evidence for the identification of the introduction vector, we infer that the most likely vector was the aquarium trade and that the Ebro Delta colonization followed a human-mediated

secondary introduction isolated from the rest of Iberia. We predict that considering the high tolerance to salinity and temperature and the significant amount of colonized area in the Iberian southern region, *F. heteroclitus* will most likely keep on expanding its invasive range until it faces unfavourable environmental conditions. Before addressing the main interpretations and conclusions of these results, one main caveat must be addressed. Successful understanding of the invasion pathway relies on (1) comparable genetic data retrieved from an adequate number of sampled individuals throughout the entire native range, (2) presence of genetic clines within the native populations and (3) the use of adequate molecular markers (Geller *et al.* 2010). While the first two points are fulfilled for *F. heteroclitus*, the use of a single mitochondrial DNA marker constitutes the main caveat of this study. Mitochondrial DNA has been a widely used molecular marker in population genetics studies (Ballard & Whitlock 2004). Nevertheless, the use of high variable nuclear markers, such as microsatellites, provides an opportunity to perform assignment tests based on their multiple-locus genotypes, to test for recent reductions in population sizes and to estimate effective population sizes. Single nucleotide polymorphisms (SNPs, Morin *et al.* 2004), extend the previously referred analytical possibilities improving their statistical power because of the sheer number of existing loci genome wide.

### **Genetic diversity**

The presence of a single haplotype common to all *F. heteroclitus* sampled in Iberia lends support to the hypothesis of an extremely recent introduction of the species which has not allowed the accumulation of mutations at the mtDNA level, and with a single introduction event composed by a very small number of individuals (Roman & Darling 2007). Theoretically, invasive species are expected to suffer loss of genetic variation since the new established populations are often based on a few individuals, which by definition, are less genetically diverse than the native the source populations (Allendorf & Lundquist 2003; Dlugosch & Parker 2008; Nei *et al.* 1975). The single-haplotype characteristic can be found in other invasive species, such as *Equulites elongatus*, the slender pony fish (Sakinan *et al.* 2017); *Cercopagis pengoi*, the fishhook waterflea, a planktonic cladoceran crustacean (Cristescu *et al.* 2001); *Corbicula fluminea*, the Asian clam (Gomes *et al.* 2016) and *Didemnum perlucidum*, a sea squirt (Dias *et al.* 2016). However, many successful invasive species do not display significant erosion of genetic diversity (Dlugosch & Parker 2008; Rius *et al.* 2014; Roman & Darling 2007). For example, according to Rius *et al.* (2014), a recent review of the literature on invasion genetics from the European seas, while ca. 73% of the studies comparing the genetic diversity

between introduced species and their native range reported comparable levels of diversity between them, only ca. 23% displayed a reduction in the genetic diversity of introduced species.

### **Population sources**

We identified a single Iberian haplotype present in all USA northern populations in high frequency (between 47 and 93%) and in two individuals in Suffolk, a southern location. According to our results, the northern group is the most probable source of the founder individuals, which corresponds to the natural range of the subspecies *F. h. macrolepidotus*. However, we cannot definitely exclude Suffolk as a presumptive population source. The absence of genetic diversity in Iberia prevents to infer, accurately, the putative source population. The low spatial resolution of our data arises from the lack of genetic variability in the invasive range, rather than insufficient sampling of *F. heteroclitus* individuals in the Iberian Peninsula or in its native range (Muirhead *et al.* 2008).

Nevertheless, our findings are consistent with two previous studies on the origin of invasive individuals found in the Guadalquivir. First, based on mtDNA restriction fragment length polymorphisms (RFLP) Fernández-Pedrosa *et al.* (1996) reported the presence of two haplotypes: the most abundant corresponding to the northern haplotype 1, dominant between Maine and Nova Scotia in North America; the other haplotype did not match any of the sampled native haplotypes and we found no evidence of its presence in the present study, using a larger number of individuals. It was previously suggested to be either a native unsampled haplotype or an endemic haplotype from Iberia, which is rather unlikely due to its recent invasion (Fernández-Pedrosa *et al.* 1996) and total absence of records in the area. Although there are no reports of hybridization between *F. heteroclitus* and any of the Spanish endemic species, the presence of a new haplotype could nonetheless be due hybridization (Rius *et al.* 2014). Secondly, a study based on *cyt b* sequences (Bernardi *et al.* 1995) concluded the individuals from the Guadalquivir originated in the region between Maine and Nova Scotia. Although our results are congruent with this proposed origin, we show that source populations could come from lower latitudes (40° N), even if only the northern group is considered.

### **Introduction vector**

Since it was first recorded in Iberian saltmarshes, several studies have linked *F. heteroclitus* introduction to different vectors. The aquarium trade has been the most suggested vector responsible for the introduction of this species (Elvira & Almodóvar 2001; García-Llorente *et al.* 2008; Gozlan 2010; Hernando & Soriguer 1992; Ribeiro *et al.* 2008a), followed by ballast



water (Fernández-Delgado 2010; García-Revilla & Fernández-Delgado 2009), biological control (Gozlan 2010) and unknown origins (Fernández-Delgado 1989; Gutiérrez-Estrada *et al.* 1998). We posit that the introduction via aquarium trade is the most probable scenario responsible for the establishment of the first individuals in Iberia, given the obtained genetic results.

It is well known that vessels can transport large numbers of organisms from several species at the same time in their ballast water (Carlton 2001; Gollasch 2007). Since there are countless vessels active around the world (e.g., Kaluza *et al.* 2010), ballast water-mediated transport potentiates multiple introduction events, each with large groups of individuals (Hulme 2009). Considering such large groups usually comprise higher genetic diversity than fewer individuals alone, the assemblages transported are likely to display levels of genetic diversity similar to the levels found within their native range (Dlugosch & Parker 2008; Roman 2006; Wilson *et al.* 2009). Our findings, however, suggest that this was not the introduction vector responsible for the spread of the species: in contrast to the expectation of similar levels of genetic diversity between the native and invasive range, the Iberian locations display a strong founder effect, with all samples sharing a single haplotype.

Similarly to ballast water, the aquarium and ornamentals trade transport many species at a global scale (Padilla & Williams 2004). For instance, at least 19% of the invasive fishes found in Iberia were introduced via aquarium trade (Maceda-Veiga *et al.* 2013). However, there are two main differences between introductions that follow ballast water or aquarium trade. First, each introduction event after aquarium release is likely to comprise a small number of individuals (Duggan *et al.* 2006; Roman & Darling 2007). Thus, even though this vector may be responsible for the establishment of several non-native species (Padilla & Williams 2004), multiple introductions would be necessary for an invasive species to display high genetic diversity (Facon *et al.* 2003; Roman & Darling 2007). Secondly, the individuals released by aquarists are usually adults of higher fitness, which makes them better adapted to survive in a natural environment (Padilla & Williams 2004). Thus, not only the *F. heteroclitus* invasive genetic diversity and structure are consistent with an introduction of a low number of individuals via aquarium trade, but it is also plausible that a few resistant individuals would manage to survive, reproduce and colonize the environment in which they were released.

### **Human mediated introduction in the Ebro Delta**

Although the absence of genetic structure within Iberia limits possible insights into the invasion pathway, our data support the hypothesis of an human-mediated introduction episode

responsible for the establishment of *F. heteroclitus* in the Ebro Delta, as previously suggested by Gisbert & López (2007) based on taxonomic identification. The hypothesis of a long-distance colonization via natural dispersal is quite unlikely.

According to a review of the geographical distribution of Cyprinodontiformes along the northeastern coast of Spain by García-Berthou & Moreno-Amich (1991), no *F. heteroclitus* individuals were found at the Ebro Delta in 1989; the first record was only registered 16 years later by Gisbert & López (2007). Thus, we estimate the date of establishment in the Ebro Delta ranges between 12 and 28 years ago. Assuming this estimate is correct, a natural colonization hypothesis implies individuals would have taken roughly two decades to travel more than 1000 km from their southernmost limit located in the Guadalquivir saltmarshes (Gutiérrez-Estrada *et al.* 1998) until the Ebro Delta. However, when compared with the natural colonization of the Ria Formosa, this hypothesis seems quite improbable. While *F. heteroclitus* was never collected during sampling events that happened in the Ria Formosa between 1980 and 2006 (França *et al.* 2009; Ribeiro *et al.* 2006; Ribeiro *et al.* 2008b), analysis of prey remains left by Little Terns (*Sterna albifrons*) in the salt-pans and barriers islands revealed this species was present at least since 2002 (Catry *et al.* 2006; Paiva *et al.* 2006a). Although this may sound contradictory, *F. heteroclitus* could in fact have been present in the Ria Formosa in specific unsampled locations or at extremely low densities, avoiding capture. Nonetheless, assuming that colonization happened around 2002, it seems that *F. heteroclitus* took no more than *ca.* 20 years to travel around 50 km from the Guadiana Delta, where it was first detected in 1976 (Coelho *et al.* 1976). This estimate indicates that if the Ebro colonization happened via natural dispersal, it must have happened 20 times faster than the natural colonization of the Ria Formosa. Given that *F. heteroclitus* has very low dispersal abilities (e.g., Fritz *et al.* 1975; Lotrich 1975), the natural colonization scenario seems unlikely.

Furthermore, if establishment in the Ebro Delta followed a natural range expansion one would expect to find several established populations between the Barbate marshes in the Guadalquivir, its southernmost location (Gutiérrez-Estrada *et al.* 1998), and the Ebro Delta. However, we know that no invasive individuals can be found at the Mar Menor (personal communication), a coastal lagoon with suitable conditions for successful establishment (Gutiérrez-Estrada *et al.* 1998; Pérez-Ruzafa *et al.* 2006), located midway between the Guadalquivir and the Ebro Delta (Figure 1.1).

Finally, the Strait of Gibraltar would represent a barrier to dispersal and gene flow, limiting *F. heteroclitus* natural range expansion towards the Mediterranean Sea (Doadrio *et al.* 2002; Fernández-Delgado *et al.* 1986; Moreno-Amich *et al.* 1999) because of the strong

currents prevailing in the area and the absence of suitable habitats. Although uncertain for *F. heteroclitus*, this has already been documented for two other Iberian toothcarps, where restricted gene flow in this region led to speciation of the *Aphanius iberus* in the Mediterranean Iberian coast and *Aphanius baeticus* in the southwestern Atlantic Spanish coast (Doadrio *et al.* 2002; Perdices *et al.* 2001).

## References

- Able KW, Felley JD (1986) Geographical variation in *Fundulus heteroclitus*: tests for concordance between egg and adult morphologies. *American Zoologist* **26**, 145-157.
- Adams SM, Lindmeier JB, Duvernell DD (2006) Microsatellite analysis of the phylogeography, Pleistocene history and secondary contact hypotheses for the killifish, *Fundulus heteroclitus*. *Molecular ecology* **15**, 1109-1123.
- Allendorf FW, Lundquist LL (2003) Introduction: population biology, evolution, and control of invasive species. *Conservation Biology* **17**, 24-30.
- Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. *Molecular ecology* **13**, 729-744.
- Bandelt HJ, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**, 37-48.
- Bax N, Williamson A, Aguero M, Gonzalez E, Geeves W (2003) Marine invasive alien species: a threat to global biodiversity. *Marine policy* **27**, 313-323.
- Bernardi G, Fernandez-Delgado C, Gomez-Chiarri M, Powers D (1995) Origin of a Spanish population of *Fundulus heteroclitus* inferred by cytochrome b sequence analysis. *Journal of Fish Biology* **47**, 737-740.
- Bernardi G, Sordino P, Powers DA (1993) Concordant mitochondrial and nuclear DNA phylogenies for populations of the teleost fish *Fundulus heteroclitus*. *Proceedings of the National Academy of Sciences* **90**, 9271-9274.
- Bigelow HB, Schroeder WC (1953) *Fishes of the Gulf of Maine*. US Government Printing Office, Washington, DC.  
[https://archive.org/stream/fishesofgulfofma1953bige/fishesofgulfofma1953bige\\_djvu.txt](https://archive.org/stream/fishesofgulfofma1953bige/fishesofgulfofma1953bige_djvu.txt)
- Bock DG, Caseys C, Cousens RD, *et al.* (2015) What we still don't know about invasion genetics. *Molecular ecology* **24**, 2277-2297.
- Carlton JT (2001) The scale and ecological consequences of biological invasions in the World's oceans. In: *Invasive species and biodiversity management* (eds. Sandlund O, Schei P, Viken A), pp. 195-212, Dordrecht: Kluwer Academic Publishers.
- Catry T, Ramos JA, Paiva VH, *et al.* (2006) Intercolony and annual differences in the diet and feeding ecology of little tern adults and chicks in Portugal. *The Condor* **108**, 366-376.
- Coelho M, Gomes J, Re P (1976) *Valencia hispanica*, a new fish to Portugal. *Archivos do Museu Bocage (2a serie). Notas e suplementos*, 1-3.
- Cristescu ME (2015) Genetic reconstructions of invasion history. *Molecular ecology* **24**, 2212-2225.
- Cristescu ME, Hebert PD, Witt JD, MacIsaac HJ, Grigorovich IA (2001) An invasion history for *Cercopagis pengoi* based on mitochondrial gene sequences. *Limnology and Oceanography* **46**, 224-229.

- Dias J, Rocha R, Godwin S, *et al.* (2016) Investigating the cryptogenic status of the sea squirt *Didemnum perlucidum* (Tunicata, Ascidiacea) in Australia based on a molecular study of its global distribution. *Aquatic Invasions* **11**, 239-245.
- Dlugosch K, Parker I (2008) Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Molecular ecology* **17**, 431-449.
- Doadrio I, Carmona JA, Fernandez-Delgado C (2002) Morphometric study of the Iberian *Aphanius* (Actinopterygii, Cyprinodontiformes), with description of a new species. *Folia Zoologica, Praha* **51**, 67-80.
- Duggan IC, Rixon CA, MacIsaac HJ (2006) Popularity and propagule pressure: determinants of introduction and establishment of aquarium fish. *Biological invasions* **8**, 377-382.
- Duvernell DD, Lindmeier JB, Faust KE, Whitehead A (2008) Relative influences of historical and contemporary forces shaping the distribution of genetic variation in the Atlantic killifish, *Fundulus heteroclitus*. *Molecular ecology* **17**, 1344-1360.
- Elvira B, Almodóvar A (2001) Freshwater fish introductions in Spain: facts and figures at the beginning of the 21st century. *Journal of Fish Biology* **59**, 323-331.
- Estoup A, Guillemaud T (2010) Reconstructing routes of invasion using genetic data: why, how and so what? *Molecular ecology* **19**, 4113-4130.
- Facon B, Pointier JP, Glaubrecht M, *et al.* (2003) A molecular phylogeography approach to biological invasions of the New World by parthenogenetic Thiarid snails. *Molecular ecology* **12**, 3027-3039.
- Fernández-Delgado C (1989) Life-history patterns of the salt-marsh killifish *Fundulus heteroclitus* (L.) introduced in the estuary of the Guadalquivir River (South West Spain). *Estuarine, Coastal and Shelf Science* **29**, 573-582.
- Fernández-Delgado C (2010) Especies introducidas en Andalucía: El caso de los peces. *Especies exóticas invasoras en Andalucía. Talleres provinciales 2004-2006*, 132-137.
- Fernández-Delgado C, Hernando J, Herrera M, Bellido M (1986) Sobre el status taxonómico del género *Valencia* Myers, 1928 en el suroeste de Iberia. *Donana, Acta Vertebrata* **13**, 161-163.
- Fernández-Pedrosa V, Latorre A, González A (1996) Evidence from mtDNA RFLP analysis for the introduction of *Fundulus heteroclitus* to southwestern Spain. *Journal of Fish Biology* **48**, 1278-1282.
- França S, Costa MJ, Cabral HN (2009) Assessing habitat specific fish assemblages in estuaries along the Portuguese coast. *Estuarine, Coastal and Shelf Science* **83**, 1-12.
- Fritz E, Meredith W, Lotrich V (1975) Fall and winter movements and activity level of the mummichog, *Fundulus heteroclitus*, in a tidal creek. *Chesapeake Science* **16**, 211-215.
- Galil B, Marchini A, Occhipinti-Ambrogi A, *et al.* (2014) International arrivals: widespread bioinvasions in European Seas. *Ethology Ecology & Evolution* **26**, 152-171.
- García-Berthou E, Moreno-Amich R (1991) New records of *Aphanius iberus* (Pisces: Cyprinodontidae) and review of the geographical distribution of cyprinodontiform fishes in Catalonia (NE-Spain). *Scientia gerundensis* **17**, 69-76.
- García-Llorente M, Martín-López B, González JA, Alcorlo P, Montes C (2008) Social perceptions of the impacts and benefits of invasive alien species: Implications for management. *Biological Conservation* **141**, 2969-2983.
- García-Revilla MG, Fernández-Delgado C (2009) *La introducción por mar de especies exóticas invasoras a través del agua de lastre de los barcos: el caso de Doñana*. Servicio de Publicaciones, Universidad de Córdoba. pp. 176. [www.uco.es/aphanius/includes/descargapub.php?id=8](http://www.uco.es/aphanius/includes/descargapub.php?id=8)
- Geller JB, Darling JA, Carlton JT (2010) Genetic perspectives on marine biological invasions. *Annual Review of Marine Science* **2**, 367-393.

- Ghabooli S, Shiganova TA, Briski E, *et al.* (2013) Invasion pathway of the ctenophore *Mnemiopsis leidyi* in the Mediterranean Sea. *PLoS one* **8**, e81067.
- Gisbert E, López M (2007) First record of a population of the exotic mummichog *Fundulus heteroclitus* (L., 1766) in the Mediterranean Sea basin (Ebro River delta). *Journal of Fish Biology* **71**, 1220-1224.
- Gollasch S (2007) Is ballast water a major dispersal mechanism for marine organisms? In: *Biological invasions* (ed. Nentwig W), pp. 49-57. Springer-Verlag, Berlin.
- Gomes C, Sousa R, Mendes T, *et al.* (2016) Low genetic diversity and high invasion success of *Corbicula fluminea* (Bivalvia, Corbiculidae)(Müller, 1774) in Portugal. *PLoS one* **11**, e0158108.
- González-Vilaseñor LI, Powers DA (1990) Mitochondrial-DNA restriction-site polymorphisms in the teleost *Fundulus heteroclitus* support secondary intergradation. *Evolution* **44**, 27-37.
- Gozlan RE (2010) The cost of non-native aquatic species introductions in Spain: fact or fiction? *Aquatic Invasions* **5**, 231-238.
- Gutiérrez-Estrada J, Prenda J, Oliva F, Fernández-Delgado C (1998) Distribution and habitat preferences of the introduced mummichog *Fundulus heteroclitus* (Linnaeus) in southwestern Spain. *Estuarine, Coastal and Shelf Science* **46**, 827-835.
- Handley L-JL, Estoup A, Evans D, *et al.* (2011) Ecological genetics of invasive alien species. *BioControl* **56**, 409.
- Hardy Jr JD (1978) *Development of fishes of the mid-Atlantic Bight*. Vol. II. *Anguillidae through Syngnathidae*. US Fish and Wildlife Service Publication FWS/OBS-78/12.
- Hernando J (1975) Nuevas localidades de *Valencia hispanica* (Pisces: Cyprinodontidae) en el suroeste de España. *Doñana Acta Vertebrata* **2**, 265-267.
- Hernando J, Sorriquer M (1992) Biogeography of the freshwater fish of the Iberian Peninsula. *Limnetica* **8**, 243-253.
- Hulme PE (2009) Trade, transport and trouble: managing invasive species pathways in an era of globalization. *Journal of applied ecology* **46**, 10-18.
- Kaluza P, Kölzsch A, Gastner MT, Blasius B (2010) The complex network of global cargo ship movements. *Journal of the Royal Society Interface* **7**, 1093-1103.
- Katsanevakis S, Crocetta F (2014) Pathways of introduction of marine alien species in European waters and the Mediterranean—A possible undermined role of marine litter, 61-68.
- Kneib RT (1984) Patterns in the utilization of the intertidal salt marsh by larvae and juveniles of *Fundulus heteroclitus* (Linnaeus) and *Fundulus luciae* (Baird). *Journal of Experimental Marine Biology and Ecology* **83**, 41-51.
- Kulhanek SA, Ricciardi A, Leung B (2011) Is invasion history a useful tool for predicting the impacts of the world's worst aquatic invasive species? *Ecological applications* **21**, 189-202.
- Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451-1452.
- Lotrich VA (1975) Summer home range and movements of *Fundulus heteroclitus* (Pisces: Cyprinodontidae) in a tidal creek. *Ecology* **56**, 191-198.
- Maceda-Veiga A, Escribano-Alacid J, de Sostoa A, García-Berthou E (2013) The aquarium trade as a potential source of fish introductions in southwestern Europe. *Biological invasions* **15**, 2707.
- Martin AP, Bermingham E (1998) Systematics and evolution of lower Central American cichlids inferred from analysis of cytochrome b gene sequences. *Molecular phylogenetics and evolution* **9**, 192-203.

- McKenzie JL, Dhillon RS, Schulte PM (2015) Evidence for a bimodal distribution of hybrid indices in a hybrid zone with high admixture. *Royal Society open science* **2**, 150285.
- McKenzie JL, Dhillon RS, Schulte PM (2016) Steep, coincident, and concordant clines in mitochondrial and nuclear-encoded genes in a hybrid zone between subspecies of Atlantic killifish, *Fundulus heteroclitus*. *Ecology and Evolution* **6**, 5771-5787.
- Meyerson LA, Mooney HA (2007) Invasive alien species in an era of globalization. *Frontiers in Ecology and the Environment* **5**, 199-208.
- Millennium Ecosystem Assessment (2005) *Ecosystems and Human Well-being: Biodiversity Synthesis*. World Resources Institute, Washington, DC. <http://millenniumassessment.org/en/Synthesis.html>
- Molnar JL, Gamboa RL, Revenga C, Spalding MD (2008) Assessing the global threat of invasive species to marine biodiversity. *Frontiers in Ecology and the Environment* **6**, 485-492.
- Moreno-Amich R, Planelles M, Fernández-Delgado C, García-Berthou E (1999) Distribución geográfica de los ciprinodontiformes en la Península ibérica. *Peces Ciprinodontidos Ibéricos: Fartet y Samaruc. Monografía*, 33-57.
- Morin PA, Luikart G, Wayne RK (2004) SNPs in ecology, evolution and conservation. *Trends in ecology & evolution* **19**, 208-216.
- Morin RP, Able KW (1983) Patterns of geographic variation in the egg morphology of the fundulid fish, *Fundulus heteroclitus*. *Copeia* **1983**, 726-740.
- Muirhead JR, Gray DK, Kelly DW, *et al.* (2008) Identifying the source of species invasions: sampling intensity vs. genetic diversity. *Molecular ecology* **17**, 1020-1035.
- Nei M (1987) Genetic distance and molecular phylogeny. In: *Population Genetics & Fishery Management* (eds. Ryman N, Utter FW), pp. 193–223. Washington Sea Grant Program, University of Washington, Seattle.
- Nei M, Maruyama T, Chakraborty R (1975) The bottleneck effect and genetic variability in populations. *Evolution* **29**, 1-10.
- Nei M, Tajima F (1981) DNA polymorphism detectable by restriction endonucleases. *Genetics* **97**, 145–163.
- Otero M, Cebrian E, Francour P, Galil B, Savini D (2013) Monitoring Marine Invasive Species in Mediterranean Marine Protected Areas (MPAs): A strategy and practical guide for managers. *Malaga, Spain: IUCN*, 136.
- Padilla DK, Williams SL (2004) Beyond ballast water: aquarium and ornamental trades as sources of invasive species in aquatic ecosystems. *Frontiers in Ecology and the Environment* **2**, 131-138.
- Paiva VH, Ramos JA, Catry T, *et al.* (2006a) Influence of environmental factors and energetic value of food on Little Tern *Sterna albifrons* chick growth and food delivery. *Bird Study* **53**, 1-11.
- Paiva VH, Ramos JA, Machado D, *et al.* (2006b) Importance of marine prey to growth of estuarine tern chicks: evidence from an energetic balance model, 241-255.
- Palumbi S, Martin A, Romano S, *et al.* (1991) *The Simple Fool's Guide to PCR, Version 2.0* University of Hawaii Press, Honolulu, HI, 96822.
- Perdices A, Carmona JA, Fernández-Delgado C, Doadrio I (2001) Nuclear and mitochondrial data reveal high genetic divergence among Atlantic and Mediterranean populations of the Iberian killifish *Aphanius iberus* (Teleostei: Cyprinodontidae). *Heredity* **87**, 314-324.
- Pérez-Ruzafa A, Garcia-Charton J, Barcala E, Marcos C (2006) Changes in benthic fish assemblages as a consequence of coastal works in a coastal lagoon: The Mar Menor (Spain, Western Mediterranean). *Marine pollution bulletin* **53**, 107-120.

- Perrings C, Dehnen-Schmutz K, Touza J, Williamson M (2005) How to manage biological invasions under globalization. *Trends in ecology & evolution* **20**, 212-215.
- Powers DA, Place AR (1978) Biochemical genetics of *Fundulus heteroclitus* (L.). I. Temporal and spatial variation in gene frequencies of *Ldh-B*, *Mdh-A*, *Gpi-B*, and *Pgm-A*. *Biochemical genetics* **16**, 593-607.
- Powers DA, Ropson I, Brown DC, *et al.* (1986) Genetic variation in *Fundulus heteroclitus*: geographic distribution. *American Zoologist* **26**, 131-144.
- Ribeiro F, Elvira B, Collares-Pereira MJ, Moyle PB (2008a) Life-history traits of non-native fishes in Iberian watersheds across several invasion stages: a first approach. *Biological invasions* **10**, 89-102.
- Ribeiro J, Bentes L, Coelho R, *et al.* (2006) Seasonal, tidal and diurnal changes in fish assemblages in the Ria Formosa lagoon (Portugal). *Estuarine, Coastal and Shelf Science* **67**, 461-474.
- Ribeiro J, Monteiro CC, Monteiro P, *et al.* (2008b) Long-term changes in fish communities of the Ria Formosa coastal lagoon (southern Portugal) based on two studies made 20years apart. *Estuarine, Coastal and Shelf Science* **76**, 57-68.
- Rius M, Turon X, Bernardi G, Volckaert FA, Viard F (2014) Marine invasion genetics: from spatio-temporal patterns to evolutionary outcomes. *Biological invasions* **17**, 869-885.
- Roderick GK, Navajas M (2003) Genes in new environments: genetics and evolution in biological control. *Nature Reviews Genetics* **4**, 889-899.
- Roman J (2006) Diluting the founder effect: cryptic invasions expand a marine invader's range. *Proceedings of the Royal Society of London B: Biological Sciences* **273**, 2453-2459.
- Roman J, Darling JA (2007) Paradox lost: genetic diversity and the success of aquatic invasions. *Trends in ecology & evolution* **22**, 454-464.
- Ropson IJ, Brown DC, Powers DA (1990) Biochemical genetics of *Fundulus heteroclitus* (L.). VI. Geographical variation in the gene frequencies of 15 loci. *Evolution* **44**, 16-26.
- Sakinan S, Karahan A, Ok M (2017) Integration of DNA barcoding for the initial recordings of Lessepsian fishes: a case study of the Indo-Pacific slender ponyfish *Equulites elongatus*. *Journal of Fish Biology* **90**, 1054-1061.
- Sambrook J, Russel DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Smith MW, Chapman RW, Powers DA (1998) Mitochondrial DNA analysis of Atlantic coast, Chesapeake Bay, and Delaware Bay populations of the teleost *Fundulus heteroclitus* indicates temporally unstable distributions over geologic time. *Molecular Marine Biology and Biotechnology* **7**, 79-87.
- Strand AE, Williams LM, Oleksiak MF, Sotka EE (2012) Can diversifying selection be distinguished from history in geographic clines? A population genomic study of killifish (*Fundulus heteroclitus*). *PloS one* **7**, e45138.
- Wallentinus I, Nyberg CD (2007) Introduced marine organisms as habitat modifiers. *Marine pollution bulletin* **55**, 323-332.
- Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* **10**, 506-513.
- Williams LM, Ma X, Boyko AR, Bustamante CD, Oleksiak MF (2010) SNP identification, verification, and utility for population genetics in a non-model genus. *BMC genetics* **11**, 1.
- Williams SL, Davidson IC, Pasari JR, *et al.* (2013) Managing multiple vectors for marine invasions in an increasingly connected world. *Bioscience* **63**, 952-966.
- Wilson JR, Dormontt EE, Prentis PJ, Lowe AJ, Richardson DM (2009) Something in the way you move: dispersal pathways affect invasion success. *Trends in ecology & evolution* **24**, 136-144.