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GENETIC DIVERSITY AND POPULATION STRUCTURE OF THE NON-NATIVE EASTERN MOSQUITOFISH (*Gambusia holbrooki*) IN MEDITERRANEAN STREAMS

David Díez del Molino

UNIVERSITAT DE GIRONA
DOCTORAL THESIS

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David Díez del Molino

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Hereby, the Professor JOSE LUIS GARCÍA-MARÍN of the UNIVERSITY OF GIRONA certifies that:

This doctoral thesis entitled ''**GENETIC DIVERSITY AND POPULATION STRUCTURE OF THE NON-NATIVE EASTERN MOSQUITOFISH** *(Gambusia holbrooki)* **IN MEDITERRANEAN STREAMS**'' that DAVID DÍEZ DEL MOLINO has submitted to obtain the doctoral degree from the UNIVERSITY OF GIRONA has been completed under my supervision, and meets the requirements to opt for the *International Doctor* mention.

In witness whereof and for such purposes as may arise, the following certification is signed:

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DAVID DÍEZ DEL MOLINO
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Contents

Abstract

Invasive species are a pressing threat to biodiversity, particularly on freshwater ecosystems. Genetic studies on invasive species had focused on identify routes of invasion and levels of diversity retained in invasive populations, but less attention has been devoted to describe fine-scale population divergences in invaded territories. Mosquitofish is one of the worldwide worst freshwater invaders. The evolutionary forces determining local divergence as well as the temporal components of genetic diversity and geographical structure of invasive mosquitofish populations are also poorly known. Such genetic information could assist to control invasive success and prevent further expansion of current populations.

Using microsatellite loci, we assessed the genetic diversity and spatial population structure of mosquitofish *(Gambusia holbrooki)* retained in invaded Spanish watersheds, when compared with the American locations close to the putative source populations. To determine temporal stability of genetic diversity patterns and dispersal rates, we analyzed four consecutive cohorts of *G. holbrooki* from three different river basins. We also analyzed genetic variation in introduced mosquitofish in a reservoir of the Ebro River in which severe chronic pollution has been well documented, to test whether fragmentation resulting from dam and pollutants can modify diversity levels and population structure at regional scales.

Introduced mosquitofish populations studied display lower levels of genetic diversity than populations at the core of the native area of distribution in North America. However, they have genetic diversity levels in agreement with those described for the postglacially colonized American sources of the European introduction. We hypothesized that European mosquitofish probably retained the evolutionary potential allocated in these sources. Genetic diversity levels were similar between locations of the study Spanish rivers, and despite evidencing inter-annual effective population size fluctuations, local genetic diversity levels were maintained among cohorts. Hydrography and connectivity were the main drivers of differentiation producing significant but temporally stable population structure among the introduced mosquitofish. Large proportions of immigrants (20-50%) contributed to local populations every year, helping to maintain levels of diversity, and facilitating the spread and colonization of suitable habitats throughout the entire river basin. As observed in the Ebro River, human disturbances as pollution or dams do not prevent mosquitofish dispersal along rivers, in fact, irrigation channels in the lowland plain of the northernmost Spanish Mediterranean rivers have the opposite effect, promoting gene flow among basins. In some river basins, a one dimensional IBD differentiation pattern arise as consequence of the natural linear fish dispersal. A two dimensional IBD pattern of population divergence observed at regional scales likely resulted from human disturbances on habitat (i.e. increasing connectivity). Unregulated human-assisted translocations increased the opportunities for colonization of new environments, and particularly the upstream reaches of the rivers where the species is currently absent.

Resumen

Las especies invasoras son una seria amenaza para la biodiversidad, especialmente en los ecosistemas de aguas continentales. La mayoría de los estudios genéticos sobre las invasiones biológicas se centran en identificar las vías de invasión y los niveles de diversidad en poblaciones introducidas. Menor atención se ha prestado a describir detalladamente los patrones de diferenciación entre estas poblaciones. La gambusia es una de las especies invasoras de aguas continentales que mayores impactos negativos produce a escala mundial. Los agentes evolutivos que determinan los patrones de diferenciación local, así como los componentes temporales de diversidad genética y estructura geográfica de las poblaciones invasoras de gambusia son sin embargo poco conocidos. Esta información podría ser crítica para controlar su éxito invasor y así prevenir futuras expansiones a zonas donde la especie aún no está presente.

En este trabajo, mediante el estudio de la variación en microsatélites, describimos los niveles de diversidad genética y los patrones de diferenciación entre las poblaciones de *Gambusia holbrooki* introducidas en cuencas españolas, y además los comparamos con aquellos obtenidas en poblaciones Americanas geográficamente cercanas a las poblaciones de origen de las introducciones. Para determinar la estabilidad temporal en los patrones de diversidad genética y dispersión, analizamos cuatro cohortes consecutivas en tres cuencas distintas (Muga, Fuvià y Ter). Finalmente, estudiamos la variación genética en y entre poblaciones de gambusia en un embalse altamente contaminado del río Ebro para comprobar si la fragmentación producida por los contaminantes o la propia presa pueden afectar los niveles de diversidad y la estructura poblacional.

Las poblaciones introducidas que hemos estudiado presentan menor diversidad genética que aquellas en el centro del rango de distribución original de la especie en Norte América. Sin embargo, sus niveles de diversidad son similares a los de poblaciones Americanas cercanas a las posibles fuentes de la introducción Europea. Pese al proceso cuello de botella asociado con su introducción, las poblaciones europeas probablemente conservan el potencial evolutivo presente en esas fuentes Americanas. Todas las poblaciones españolas estudiadas presentaron similares niveles de diversidad resultaron similares y, pese a las fluctuaciones interanuales en sus tamaños efectivos, estos niveles se mantuvieron estables entre cohortes. La hidrografía y la conectividad entre las poblaciones son los principales agentes responsables de una significativa diferenciación poblacional que se mantuvo estable en el tiempo. La gran proporción de individuos inmigrantes (20-50%) contribuye a mantener los niveles de diversidad local y facilita la colonización de nuevos hábitats idóneos a lo largo de toda la cuenca fluvial. Los resultados obtenidos en el río Ebro indican que las perturbaciones humanas (contaminación o presas) no evitan en modo alguno la capacidad de dispersión de la especie, de hecho, los canales de regadío en las zonas bajas de los ríos mediterráneos del NE Español tienen un efecto contrario, favoreciendo el flujo génico entre cuencas. En algunas algunos ríos se detectaron patrones unidimensionales de aislamiento por distancia (IBD) como consecuencia de la dispersión natural lineal a lo largo del río. Sin embargo, a escala regional aparece un patrón de IBD bidimensional probablemente causado por las perturbaciones humanas sobre el hábitat que favorecen la conectividad y la translocación de individuos entre cuencas. Estas translocaciones incontroladas podrían aumentar las oportunidades para colonizar áreas donde esta especie aún no esta presente, como las zonas altas de los ríos.

Resum

Les espècies invasores són una creixent amenaça per a la biodiversitat, particularment en els ecosistemes d'aigua dolça. Els estudis genètics en espècies invasores s'han centrat en identificar les rutes d'invasió i els nivells de diversitat retinguts per les poblacions invasores, essent menys freqüents aquells treballs dedicats a descriure les divergències poblacionals a escales geogràfiques regionals dins dels territoris envaïts. La gambúsia és un peix americà que arreu ha esdevingut una de les pitjors espècies invasores d'aigua dolça. En els territoris envaïts per la gambúsia, són encara poc conegudes les forces evolutives que determinen la divergència local així com els components temporals de diversitat genètica i d'estructura geogràfica entre les poblacions. Aquesta informació genètica podria ajudar-nos a controlar l'èxit invasiu d'aquesta espècie i impedir la seva expansió cap a noves poblacions allunyades de les ja colonitzades.

Analitzant la variació en els loci microsatèl·lis, en aquest treball hem avaluat els nivells de diversitat genètica i l'estructura poblacional de la gambúsia *(Gambusia holbrooki)* present en les conques espanyoles envaïdes, comparant-los amb aquells descrits a los potencials poblacions font americanes. La estabilitat temporal dels patrons regionals de diversitat i de dispersió s'ha fet a partir de les anàlisis genètiques sobre quatre cohorts consecutives de *G. holbrooki* a tres rius (Ter, Fluvià i Muga). Hem estudiat també la variació genètica dins i entre localitats al voltant de l'embassament de Flix en el riu Ebre on hi ha ben documentada una severa i crònica contaminació. Aquestes anàlisis a Flix pretenien comprovar fins a quin punt la presa i els contaminants podrien modificar els nivells de diversitat i l'estructura població a nivell regional.

Les poblacions introduïdes de gambúsia estudiades en aquest treball presenten nivells més baixos de diversitat genètica que les poblacions al nucli de l'àrea nativa de distribució a Amèrica del Nord. Els seus nivells de diversitat genètica són però semblants a aquells descrits en les poblacions americanes en les zones de colonització post-glacial i que són properes geogràficament a les poblacions fonts utilitzades per a la introducció europea de l'espècie. En aquets treball presentem la hipòtesi de que, malgrat el possible coll d'ampolla associat amb la introducció, les poblacions europees de gambúsia probablement mantenen el potencial evolutiu present en aquelles fonts. Els nivells de diversitat genètica han estat similars entre les diferents localitats estudiades en els rius espanyols i, malgrat fluctuacions interanuals en la grandària efectiva de les poblacions, van mantenir-se estables entre cohorts. La hidrografia i la connectivitat són les principals responsables de la significativa diferenciació poblacional, aquesta diferenciació es va mantenir també estable en el temps. A les poblacions de gambúsia estudiades, cada any podem trobar una gran proporció d'immigrants (20-50% dels exemplars capturats) que ajuden a mantenir els nivells de diversitat local, i faciliten l'expansió i colonització d'hàbitats adequats al llarg de tot el riu. Els resultats a Flix suggereixen que les pertorbacions dels rius per causes antròpiques com ara la construcció de preses o la contaminació no impedeixen la dispersió de les gambúsies. De fet, els canals de irrigació poden permetre el flux gènic entre poblacions dels diferents rius tal i com hem comprovat a la plana de l'Empordà entre els rius Ter, Fluvià i Muga. En alguns dels rius estudiats hem observat un patró unidimensional d'aïllament per distància (IBD) entre les poblacions que probablement és la conseqüència de la dispersió lineal dels peixos al llarg del riu. A escala regional, s'obté un patró bidimensional de IBD que és probablement el resultat de les pertorbacions humanes que augmenten la connectivitat fins i tot entre poblacions de diferents rius. Aquestes translocacions no autoritzades d'exemplars augmenten també les oportunitats per colonitzar nous entorns, particularment nous transsectes de riu aigües amunt als actualment colonitzats per l'espècie.

1

Introduction

1.1   Biological invasions

Invasive species are considered to be one of the main threats to conserve worldwide biodiversity following habitat loss and fragmentation (UNEP, 2007), and one of the main contributors leading to global change (Novak, 2007). After human-mediated introductions into new environments beyond their natural native territories, invasive species negatively impact local fauna and ecosystems. Despite some huge control efforts, biological invasions are an emergent biological and economical concern that, far from decreasing, is growing fast (Levine, 2008). In the past 30 years, in parallel with the increase of international human travelling and trading (Perrings et al., 2002), the number of introduced species has almost doubled worldwide (Gozlan, 2008). The naturalization and incorporation of invasive species into new territories often result in ecosystem malfunctioning and detrimental interactions with native species that, in some instances, have led to the extinction of native biota (Vitousek et al., 1997; Gozlan, 2008).

Biological invasions also represent non-depreciable economic losses in many countries. Pimentel et al. (2001) estimated the joint economic damage for six nations (United States, United Kingdom, Australia, South Africa, India and Brazil) to be US\$ 336 billion per year without even considering the impacts derived from local species extinctions. According to these authors, the estimated economic damage from fish introductions in freshwater ecosystems was close to US\$ 1 billion in the United States only, even taking into account the economic benefits related with sport fishing of some introduced species (Pimentel et al., 2001). However, despite the biological and economical detrimental effects on invaded territories, most of the implications of species introductions are still unknown or poorly analyzed (Gozlan et al., 2010).

Until recent times, the research on biological invasions was mainly focused on understanding the ecological consequences of invasions and less attention has been devoted to discovering the evolutionary mechanisms that underlies the success of invasions (Lee, 2002; Allendorf and Lundquist, 2003; Novak, 2007). In addition, despite being one of the main worldwide conservation threats, biological invasions are increasingly recognized as opportunities for basic evolutionary research (Sakai et al., 2001; Lambrinos, 2004). Introduced populations often represent natural evolutionary experiments where genetic changes quickly accumulated. Through invasions, evolutionary processes like hybridization and selection can be studied at surprisingly short time

scales (Lambrinos, 2004). By incorporating evolutionary genetics to the study of biological invasions, we are creating powerful tools that are useful for revealing the main traits involved in the invasion success (Lee, 2002).

Biological invasions represent a particularly stressing problem for freshwater ecosystems (Gozlan, 2008). Fishes are both among the most introduced species around the world and among the most threatened (IUCN, 2013). In areas such as North America and central Europe, freshwater environments have been reported to be severely affected by invasions mainly associated with an increase of inland navigation routes (Ojaveer et al., 2002; Clavero et al., 2004; Ribeiro et al., 2008). In fact, many authors have positively related the increase in activities such as navigation, recreation, and river impoundment with the presence of freshwater invaders (Clavero et al., 2004; Ribeiro et al., 2008).

The Iberian Peninsula, considered a hotspot of biodiversity (Médail and Quézel, 1999), shows a large number of endemic species linked to freshwater ecosystems (Clavero et al., 2004). For example, the freshwater habitats of the autonomous community of Galicia (NW Spain) are recognized to hold a very rich aquatic fauna of both invertebrates and vertebrates (Cobo et al., 2010). Similarly, the upper reaches of the northeastern Iberian rivers still preserve the best *genetic reservoirs* of feral brown trout, but nowadays they are threatened by competition and hybridization with released non-native trout stocks (Araguas et al., 2009). Due to historical isolation, freshwater inhabitants of the Iberian Peninsula are commonly endemic (García-Berthou et al., 2000), making Iberian freshwater ecosystems even more fragile environments when facing the threat of invasive species (Elvira, 1995).

While Iberian westernmost basins (e.g., Galicia) keep their rivers with a relative reduced number of invasions (Cobo et al., 2010), other inland freshwater ecosystems have been widely invaded since the beginning of the 20th century. In fact, the number of established invasive species is higher than the native ones in many of them (García-Berthou et al., 2000). In the northeastern watersheds, for example, the fish communities are more similar to those in France than from other basins of the Iberian Peninsula, and these similarities are related with introduction routes from Europe (Clavero and García-Berthou, 2006). Moreover, aquarium trade has been identified as one of the main reasons for freshwater invasions increasing in the Iberian Peninsula (Strayer, 2010; Maceda-Veiga et al., 2013).

1.2   The genetic basis of biological invasions

Genetic variation among populations is intimately involved in the success of biological invasions (Lee, 2002). Reproductive strategies, population dynamics, environmental tolerance, and other intrinsic traits of the species are determinants to the probability of a species becoming invasive (Stepien et al., 2005). The intra-specific diversity that rules this probability should be identified to define the evolutionary factors that explain the invasion success at the population level (Guillemaud et al., 2011), and thus, whether an invasive species ultimately will establish and spread (Sakai et al., 2001).

Although the typical invasion process has many steps, it often begins with a reduced number of individuals transported from their native range to a new environment (Figure 1.1). The size of that group of individuals represents the propagule pressure of the invader upon the ecosystem. Given the general stochastic nature of introductions, this pressure is usually low (Levine, 2008). In addition, these immigrants will be exposed to new environmental pressures, likely making them
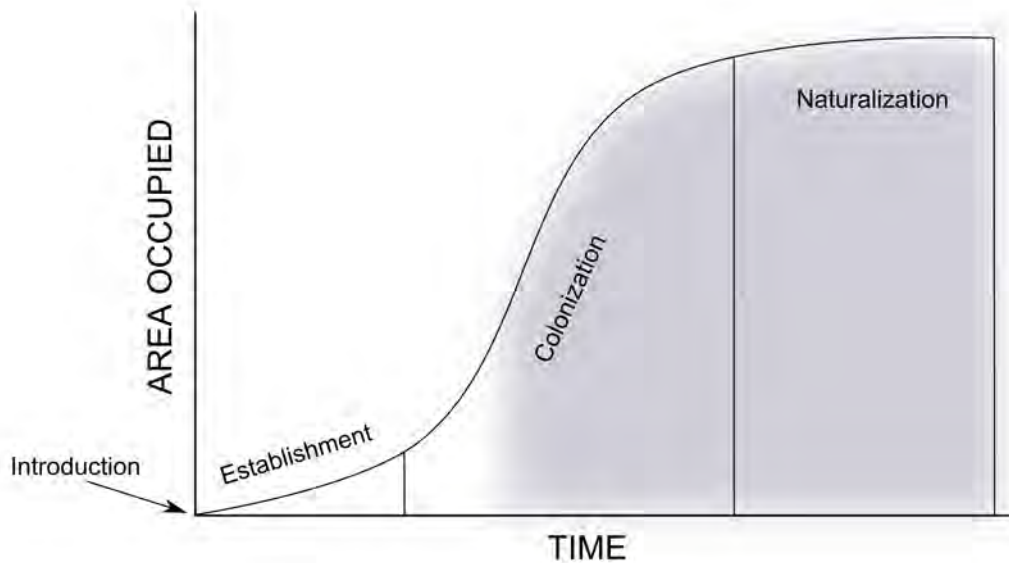
Figure 1.1: Schematic representation of a common invasion process. Lapses of time are highly variable among species and at different ecosystems. Shaded grey area indicates the species producing negative impacts at the new environment and, thus, becoming invasive (see text).

prone to high mortality rates. Therefore, a very small number of individuals survive in the new environment. That population could only be nominated as *invasive* if, after an undefined number of generations, individuals begin to be highly fecund, ecological aggressive, and spread easily, causing damages to the new environment (Sakai et al., 2001).

If only a small fraction of a source population is effectively contributing to the new population when a species is introduced, then, just by chance, a sampling effect would lead this population to have only a small representation of the genetic variability of the source population (Frankham, 2005). In addition, the newly founded population will have small number of reproductive individuals further reducing genetic diversity. These bottlenecked founding populations are also commonly exposed to genetic drift and increased inbreeding, depressing genetic diversity even further (Nei et al., 1975; Lambrinos, 2004). Therefore, in general, reduced genetic diversity is expected during the first stages of the invasion (Lockwood et al., 2005; Roman and Darling, 2007).

However, several species have shown high values of genetic diversity in the medium to later stages of the process (see Dlugosch and Parker, 2008). This phenomenon, which implies that populations that have suffered a strong decrease in effective numbers due to founder events still can maintain relatively high values of genetic diversity compared with source populations, has been called *invasion paradox* (Frankham, 2005; Roman and Darling, 2007). Several studies have already addressed the paradox. For example, Sax and Brown (2000) addressed the topic, concluding that it can be explained by well-known ecological and evolutive processes such as the stochastic nature of environments, pre-adaptation to human-disturbed environments, the absence of specifically adapted antagonist species in the new environments, the role of dispersal dynamics, and the historical contingency of evolution. So, in the end, they determined that such an invasion paradox does not exist.

Independent of the existence, or lack thereof of such a paradox, several studies on invasive species have been focused in clearing up the reasons for the maintenance -or recovery - of genetic diversity following introductions (Allendorf and Lundquist, 2003; Hufbauer, 2008). In 2004, Kolbe et al. based their studies on a lizard introduced worldwide, the brown anole (*Anolis sagrei*), and proposed that multiple introductions are not only a common phenomenon among introduced populations but also one of the main factors responsible for the maintenance of or increase in their genetic diversity. The reasons for paradoxical levels of genetic diversity, however, are highly variable among species. It has been explained by means of other evolutionary mechanisms such as hybridization (Vilà et al., 2000; Lambrinos, 2004), rapid evolutionary events (Stockwell et al., 1996), or translocations within the introduced range (Stockwell and Weeks, 1999). However, multiple introductions always represent one of the critical reasons for high levels of genetic diversity within introduced populations (Tsutsui et al., 2000; Lindholm et al., 2005; Kelly et al., 2006; Lavergne and Molofsky, 2007; Facon et al., 2008; Hufbauer, 2008; Keller and Taylor, 2010), especially in aquatic environments such as freshwater ecosystems (Roman and Darling, 2007) in which natural dispersal between newly established populations is commonly restricted.

One of the main difficulties when comparing genetic diversity between native and introduced populations is to locate the correct source populations, but this is especially complicated if the source is a *ghost population* that has never been sampled (Estoup and Guillermaud, 2010). Therefore, identifying the sources and reconstructing pathways and colonization routes are critical to address the fundamental questions of invasive populations (e.g. Vidal et al., 2010; Sanz et al., 2013) as well as to help in the design of proper management programs to eradicate or control them (Wilson et al., 2009). Multiple introductions can act as triggers of the invasion process, but successful invasions can also be originated from an intermediate and particularly successful population that has been established previously and from which several introductions can be, at the time, successful. This intermediated population would function as an *invasive bridgehead*, facilitating the colonization and spread of the species to remote, newly invaded regions (Lombaert et al., 2010). In Guillemaud et al. (2011) the authors explained that the bridgehead population scenarios are more parsimonious than those involving multiple introductions because in the first ones only the population acting as a bridgehead has to suffer the necessary evolutionary changes toward invasiveness.

1.3   *Gambusia holbrooki*

One of the most widely introduced freshwater species is the eastern mosquitofish, *Gambusia holbrooki*. This species has been introduced in river basins, marshlands, lagoons, and reservoirs worldwide and nowadays is considered to be one of the worst invasive fishes (Pyke, 2005). Established populations of the mosquitofish in Europe, Africa, Asia, and Australia have promoted local extinction and the decline of several native species (Rincón et al., 2002; Alcaraz et al., 2008; Pyke, 2008). In this work, introduced populations of *Gambusia holbrooki* from Mediterranean streams were studied to discover some of the aspects that drive their successfully invasive life history, but also as a model to analyze the wide panel of evolutionary mechanisms that may also be relevant for other freshwater invaders in their own invasive processes.

Gambusia holbrooki is included in the family Poeciliidae, characterized by the presence of the *gonopodium*, a modification of the anal fin that allows males to internally impregnate females, which also have some reproductive modifications towards ovoviviparism (Rosen and Bailey,

Figure 1.2: Graphical representation of two individuals of *G. holbrooki*. Up: female. Down: male. Note the obvious sexual dimorphism with bigger females and males presenting a fusion and elongation of three spines of the anal fin as an impregnating organ, the *gonopodium*. Art: Osado ⓑ Ⓢ Ⓞ

1963) (Figure 1.2). Due to its reproductive traits, *G. holbrooki* is a species commonly used as a model organism for experimental studies of natural and sexual selection and life-history evolution (Meredith et al., 2010).

While the study of the biogeographical history of the family is largely beyond the objectives of this thesis, it is worthwhile to briefly outline the major key points to place our species of interest in an evolutionary context. Most species of the family have vicariant distributions between South America, the Caribbean, Central America, and North America, probably as a consequence of the geological processes of separation during Cretaceous and limited dispersal after the reconnection of the Panama isthmus at the Pliocene. As consequence, Poeciliid geographical areas of distribution are largely occupied by monophyletic lineages, although some South American groups are paraphyletic, and genera such as *Poecilia* and *Gambusia* are widespread (Hrbek et al., 2007). The less specialized species of the family are classified in the *gambusinii* group. The genus *Gambusia* consists of 45 species that are ubiquitously distributed on islands, and in gulfs and coastal areas from Colombia to the northeastern United States. A study based on mtDNA concluded that the phylogenetic relationships within the genus are highly complex, but *Gambusia holbrooki* and their most close related species (*G. affinis, G. geiseri, G. heterochir* all of them distributed in North America) are a monophyletic group (Lydeard et al., 1995).

Historically, there are two major difficulties involved in accurately solving the evolutionary

relationships among species of the genus *Gambusia*. First, there is a relative lack of genetic and morphological boundaries between them, and this absence of robust definitions has led to the species suffering continuous changes of nomenclature with abundant synonymies (see Vidal et al., 2010). And second, the closeness of geographical ranges of distribution among these species, often overlapped, likely promote hybridization processes and produce individuals with intermediate morphological characteristics. In fact, episodes of introgression have been continuously documented in areas of contact of the geographical distributions among *G. holbrooki*, *G. affinis* and *G. heterochir* (Scribner and Avise, 1993; Davis, 2004).

There exists a close relation between *G. holbrooki* and *G. affinis*. In their native range, both species are continuously distributed in a broad path from the southeast to northeast regions of North America. Along this path, *G. affinis* populations are allocated to the west and so are denominated *western mosquitofish*, while *G. holbrooki* populations are located to the east, and so are denominated *eastern mosquitofish* (Wooten et al., 1988) (Figure 1.4). Scribner and Avise (1993, 1994) observed strong male-mediated introgressive hybridization favoring *G. holbrooki* genotypes, but they also detected that directional genetic flow and reproductive selection via pressure against recombinant individuals tended to dilute the effects of hybridization.

In fact, the worldwide distribution of *G. affinis* and *G. holbrooki* had been largely unclear until a few years ago, mainly because of taxonomic confusion (Pyke et al., 2008; Vidal et al., 2010). Since the early 20th century the three species *G. patruelis*, *G. affinis*, and *G. holbrooki* were introduced worldwide in order to control mosquito proliferation (Krumholz, 1948). Now *G. patruelis* is considered a synonym of *G. affinis*. On the other hand, *G. holbrooki* and *G. affinis* were classified as subspecies of *G. affinis* until Wooten et al. (1988) renamed them as two separate species. Therefore, many records of introduced *G. affinis* are actually referring to *G. holbrooki* populations (Haynes and Cashner, 1995). There is a growing panel of studies that have clarified the areas where each of these species was introduced (Vidal et al., 2010; Ayres et al., 2010; Vidal et al., 2012; Purcell et al., 2012; Sanz et al., 2013) although a worldwide range study on this matter may still needed. In Vidal et al. (2010) and Sanz et al. (2013) the authors identified that only *G. holbrooki* was introduced in European populations, including all the Spanish basins.

G. holbrooki is a lecithotrophic, life-bearing species. Males have a modification of an elongation of rays three to five of the anal fin as an impregnating organ, or *gonopodium*, used to internally impregnate females, who can store and preserve functional sperm within their reproductive tracts for months (Constantz, 1989). Contrary to what is common in Poecillids, gravid *Gambusia* females do not display superfetation (Meredith et al., 2010); instead, they can reproduce in variable lapses of time between 23 and 75 days, depending on the species and the geographical range (Krumholz, 1948; Fernández-Delgado, 1989). Mosquitofish are very fecund, with 20 to 50 offspring on average, but up to 120 per brood (Krumholz, 1948; Fernández-Delgado, 1989). In *G. holbrooki*, reproduction occurs between spring and late summer, and stops during winter. Long photoperiods and warm waters stimulate the gonadal development in *G. affinis* (Cech et al., 1992), and similar mechanisms seem to be acting in *G. holbrooki* (Carmona-Catot et al., 2013). Another reproductive characteristic of *G. holbrooki* is multiple paternity. In fact, in its native range of distribution, close to 90% broods were multiple sired (Chesser et al., 1984; Zane et al., 1999; Neff et al., 2001).

In the Iberian Peninsula, *G. holbrooki* population dynamics are well described. Briefly, these introduced populations are characterized by a spawning period from spring (mid-May) to late summer (mid-September). Thus, overwintering adult individuals from the previous year

spawn during the spring, and their generation substitution is produced by the high somatic costs of reproduction, predation, and/or illness, which lead the individuals to die after they breed (Fernández-Delgado, 1989; Cabral, 1999; Perez-Bote and Lopez, 2005). The newborns reach maturity in a few weeks and breed during summer. The offspring from this summer spawning cohort are the ones that have to survive the winter. Only a fraction of them will do so and then breed in the spring of the next year (Reznick et al., 2006b).

A wide record of introductions and negative impacts

Both mosquitofish -*G. holbrooki* and *G. affinis* - introductions were supported by governmental health agencies to control mosquito populations as vectors of diseases such as malaria (Meffe et al., 1989). To date, both species have been introduced in more than 50 countries and on all continents but Antarctica (García-Berthou et al., 2005). In Europe, introductions began with a few *G. holbrooki* individuals introduced in eastern Spain in 1921 (Krumholz, 1948; Navarro-García, 2013). From there, humans introduced the species into Italian watersheds and many other Mediterranean countries (Figure 1.3) (García-Berthou et al., 2005). In 1924, mosquitofish from Italy were introduced to the Transcaucasian regions and from there to areas in the south and center of the former USSR (Sella, 1929). Later, eastern mosquitofish were introduced to other malaric areas of the world, including East Asia and Australia (Ronchetti, 1968). As previously mentioned, due to taxonomic confusion, some of the reports of the presence of *G. affinis* are actually referring to *G. holbrooki* and vice-versa. For example, both species were reported to have been introduced in Australia, but apparently now only *G. holbrooki* is present (Congdon, 1995). Conversely, there is only the presence of *G. affinis* in New Zealand, and its populations seem to proceed from the populations of Hawaii (Purcell et al., 2012), where the species was previously introduced in 1905 (Stearns, 1983). Some genetic research has been directed towards identifying areas of precedence of individuals introduced everywhere, and the patterns detected broadly agree with historical records (e.g. Grapputo et al., 2006; Vidal et al., 2010). However, there is little precise data regarding putative American source populations, as well as a lack of well-known pathways of introduction in most of areas where the species is invasive.

Mosquitofish have been extremely successful in new environments (Meffe et al., 1989). Intense predatory activity on insect larvae and on zooplankton has sometimes altered the biological equilibrium of water systems, contributing to eutrophization (Grapputo et al., 2006). In fact, together *G. holbrooki* and *G. affinis* are considered to be one of the worst invasive species (Pyke, 2005; Alcaraz et al., 2008). Established populations of mosquitofish in Europe, Africa, Asia, and Australia have promoted the decline in and local extinction of several native species including fishes and amphibians (Rincón et al., 2002; Alcaraz et al., 2008; Pyke et al., 2008; Stockwell and Henkanaththegedara, 2011). For example, in the Iberian Peninsula, *G. holbrooki* is responsible for the reduction of feeding rates and reproductive success of *Valencia hispanica* and *Aphanius iberus*, two endemic fish species that competitively displace (Rincón et al., 2002; Carmona-Catot et al., 2013).

Population genetics of G. holbrooki

The genetic population patterns of *G. holbrooki* have been mainly studied within its native range of distribution, but during the last 40 years it has also received attention in most non-native areas of distribution where it begin to be studied as an invasive species. Gene flow patterns, genetic
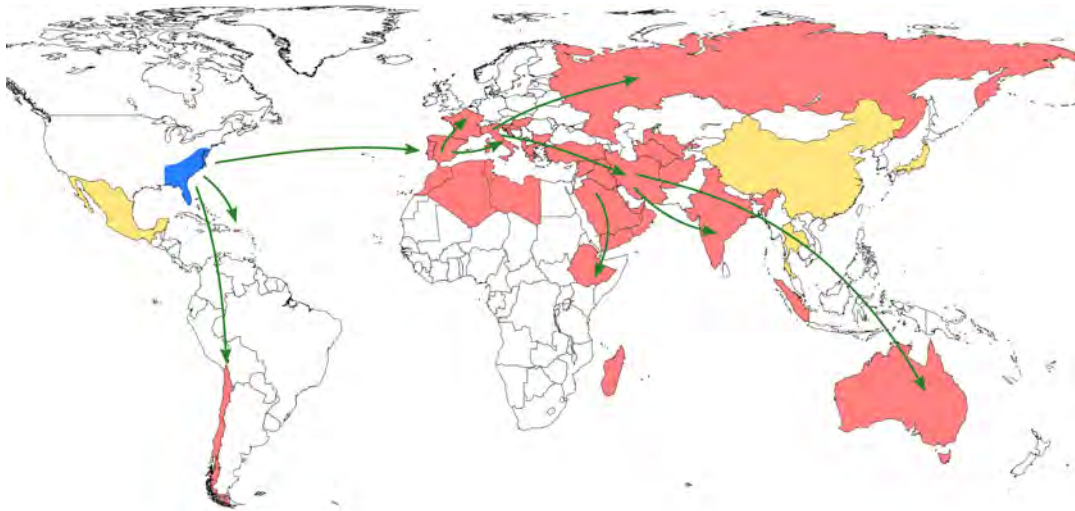
Figure 1.3: Principal introduction routes of *G. holbrooki* suggested by historical records. Blue area indicates native range of distribution. Invaded countries are red colored. Yellow colored countries are areas where the mosquitofish introduced population belonging to *G. holbrooki* or *G. affinis* is still discussion. Major routes of introduction are depicted with green arrows

diversity levels, and population structure are the most studied aspects of the species' population genetics because of their relevance in the assessment of mosquitofish invasiveness.

G. holbrooki displays high allozyme population genetic diversity in native areas of distribution (McClenaghan et al., 1985). In fact, *G. holbrooki* populations northward of the Savanah River, in the US, have high levels of genetic diversity compared to those observed for vertebrates in general, and for fishes in particular (Nevo et al., 1984; Hernandez-Martich and Smith, 1990). Based on allozyme and mtDNA differentiation, two types of *G. holbrooki* have been proposed, reflecting northward and southward allele distributions relative to the Savannah and Altamaha drainages on the Atlantic coast of the US (Wooten et al., 1988; Scribner and Avise, 1993; Hernandez-Martich et al., 1995) (Figure 1.4). The northern and less variable *G. holbrooki* populations were termed the Type I group, while the southern more variable *G. holbrooki* populations were grouped as Type II. Scribner and Avise (1993) suggested that the northern Type I emerged after colonization from southern drainages, which acted as refuge during the last glacial period.

Introduced populations tend to be less diverse than native populations as a consequence of reduced effective numbers following the introduction (Roman and Darling, 2007). This pattern has been reported for *G. holbrooki* introduced to Europe (Grapputo et al., 2006; Vidal et al., 2010; Sanz et al., 2013) and to Australia (Congdon, 1995; Ayres et al., 2010). Similar patterns are shown by *G. affinis* introduced in New Zealand (Purcell et al., 2012). However, there are also remarkable discrepancies. Introduced Hawaiian populations of *G. affinis* displayed different alleles than source populations (Scribner et al., 1992). In a comparative SNP survey, higher diversity levels were detected in European populations of *G. holbrooki* (Vidal et al., 2012).

Mosquitofish population booms in spring and summer during the reproductive season and then declines dramatically in winter (Krumholz, 1948; Pyke, 2005). Although these fluctuations tend to reduce effective numbers and levels of genetic diversity, the high reproductive potential combined with female sperm storage and multiple paternity are resources that allow mosquitofish to prevent
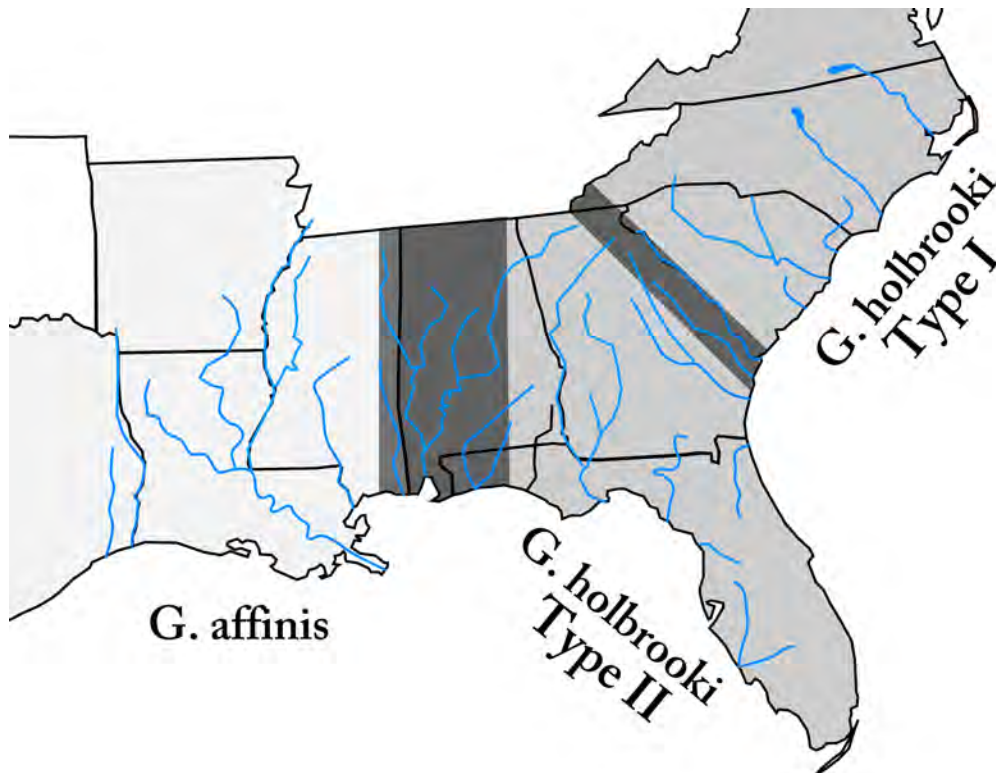
Figure 1.4: Schematic map of the areas where *G. holbrooki* is native in the east coast of North America. Areas occupied by *G. affinis, G. holbrooki* Types I and II as described by Scribner and Avise (1993). Areas of admixture between types and species are shown in dark grey.

such losses (Echelle et al., 1989; Zane et al., 1999; Spencer et al., 2000). The diversity patterns of *G. holbrooki* usually include more variable populations situated at the lower parts of the rivers. Two phenomena have been related with this pattern. First, contacts among populations are easier in downstream parts of the rivers than in upstream reaches. For example, areas of marshland at the mouths of rivers are optimal habitats for the species and allow them to disperse between drainages, especially when inundated (Congdon, 1995). And second, a pattern of directional downstream gene flow towards the lowlands of rivers as a consequence of water direction and individual transport during flood events (Congdon, 1995; Hernandez-Martich and Smith, 1997).

Native populations of *G. holbrooki* showed a clear genetic heterogeneity at short distances, which has been attributed to different evolutionary and ecological aspects: selection (Yardley et al., 1974), reproductive characteristics (Robbins et al., 1987), and stochastic factors (Smith et al., 1983; McClenaghan et al., 1985). However, most of the genetic differences among populations of this species are generally attributable to local differentiation (Hernandez-Martich and Smith, 1990). In the drainages of Savannah and Altamaha rivers on the east coast of the US, genetic diversity is distributed within rather than among populations (McClenaghan et al., 1985). These local patterns of high differentiation at short distances may promote selection to act upon geographically isolated populations (Wooten et al., 1988). Similarly, negative spatial autocorrelation at short distances has been observed as a consequence of microgeographical adaptation Kennedy et al. (1986). Along a river basin, complex microgeographical patterns

of population structure in the *Gambusia* species arise from interactions between demographic fluctuations and breeding structures complicated by multiple inseminations and sex- and cohort-specific dispersal ability Kennedy et al. (1986), but generally, local samples represent single breeding units (McClenaghan et al., 1985).

Neighboring populations are expected to be more similar to each other than to distant ones, depending on the dispersal ability of the species in a pattern of differentiation through isolation-by-distance (IBD) (Wright, 1943). Depending on whether dispersal occurs only between adjacent populations or in a more complex pattern, IBD can be a one-dimensional or two-dimensional stepping-stone model, respectively (Slatkin, 1993). For *G. holbrooki*, IBD patterns of differentiation have been detected within rivers as dispersal occurs only between adjacent populations linearly distributed in a river (Hernandez-Martich and Smith, 1997). So, the within-river differentiation pattern may be better represented by a one-dimensional stepping-stone model. Source-sink dynamics have also been proposed to influence the population structure in *G. holbrooki* (Smith et al., 1983; McElroy et al., 2011). As already stated, higher genetic diversity levels in lowland than in upstream populations suggests that unidirectional dispersal towards the downstream direction is a common pattern in the species (Hernandez-Martich and Smith, 1997). Similar tendencies have been described for guppies in Trinidad, West Indies (Shaw et al., 1994).

Dispersal ability models the levels of gene flow among populations (Hernandez-Martich and Smith, 1997). It influences population structure dynamics and plays a relevant role in both the distribution and abundance of *G. holbrooki* and thus is a key factor when determining invasion success (Endler, 1977; Sakai et al., 2001). The direction and velocity of water flow, age, and sex influence individual movement in the species (Robbins et al., 1987; Congdon, 1995; Rehage and Sih, 2004). Mosquitofish move in cohesive groups (Maglio and Rosen, 1969), but adult females and especially young females are more prone to disperse than males (Robbins et al., 1987; Rehage and Sih, 2004). However, the effects of those factors are still under discussion. For example, (Alemadi and Jenkins, 2007) did not detect differential dispersal traits for male or female *G. holbrooki*. In unimpeded corridors, this species has been estimated to be able to disperse great distances ($\sim$800 m/day) and this ability seems to be favored by deeper (<24 mm) rather than shallower waters (Alemadi and Jenkins, 2007). Similarly, Lynch (1988) detected that they can move up to 18 km a year, but twice of distance in the downstream direction than in the upstream direction. However, these high rates of dispersal can be also the exception, and not the rule (Pyke, 2005). Often, dispersal is treated as a homogeneous behaviour, but Kot et al. (1996) pointed that dispersal largely depends on the underlying individual behaviours as shy-bold dynamics (Fraser et al., 2001).

In general, introduced populations of *G. holbrooki* have high levels of spatial genetic structuring (Congdon, 1995; Ayres et al., 2010). In Australian *G. holbrooki*, genetic structures between populations were associated with the river basins from which the fish originated rather than with geographic distance (Congdon, 1995; Ayres et al., 2010). These patterns imply that despite the extent of within-river gene flow, microgeographical adaptations and temporal isolation also play an important role in population structure in introduced populations of *G. holbrooki*. Within-river, IBD patterns of differentiation have also been detected in introduced populations (Ayres et al., 2010). However, this pattern may be enforced by other factors such as physical river boundaries confounding the true contribution of distance to differentiation in invasive populations (Vidal et al., 2012).

Some studies have directly or indirectly estimated patterns of gene flow of *G. holbrooki* in

areas where the species is invasive. For example, Congdon (1995) determined that *G. holbrooki* from southern Australia display similar dispersal characteristics than in populations from native areas of distribution, with unidirectional individual movement towards downstream. Apparently, dams favor that pattern because they act as physical barriers impeding the upstream movement of individuals and facilitating upstream populations to be isolated and suffer genetic drift as consequence of founder events. Thus, these dynamics result in less diverse upstream *G. holbrooki* populations. More recently, (Ayres et al., 2010) also detected some interesting patterns of gene flow among introduced populations in Australia. First, the population structure of introduced *G. holbrooki* is strongly determined by levels of within-river dispersal. Second, areas of marshland and lowlands are often inundated, allowing the species to disperse over greater distances. And third, some populations were genetically dissimilar to populations from the same river basins but similar to those from other basins, likely reflecting human-mediated dispersal (Ayres et al., 2010).

2

Objectives

There is little data of fine-scale population genetic structure on *G. holbrooki* in invaded territories, specially in Europe. The evolutionary forces determining local levels of diversity and population divergences are also poorly known. Such genetic information can assist to control invasive success and to prevent expansion of current mosquitofish populations. Therefore, the aim of this doctoral thesis is to determine patterns of genetic diversity and population structure at fine-spatial and temporal scales in invaded Mediterranean streams to address the following primary questions:

1. Are levels of genetic diversity in the study invaded territories lower than in original sources?

2. Are observed patterns genetic diversity and population differentiation consistent over time?

3. Which agents were major responsible for the observed population structure within and among river basins?

4. Can human-made river disturbances modify patterns of genetic diversity and prevent or promote gene flow and mosquitofish expansions?

3

Methods

The main objectives of this thesis where addressed in three separated chapters, each of one them structured as scientific article. Thus, although the specific procedures and methodologies followed are explained with detail in each chapter, it is worth to briefly summarize those that are common among them.

3.1   Sample collection

In all sites, sampling areas were shallow parts along the riverbank, with low water velocity and dense vegetation. In total, 1727 individuals of *G. holbrooki* where analyzed in the studies that compose this thesis. The origin of those individuals was variate:

1. Empordà region: Most of fish were collected between 2010 and 2011 along three watersheds (Muga, Fluvià, and Ter rivers) in northeaster Spain. The Ter River has a basin area of 2955 km^2, its headwaters in the Pyrenees, and the upper course being partially snow-fed. The Fluvià (974 km^2) and Muga (758 km^2) rivers are typical Mediterranean streams with smaller watersheds and headwaters located in mountainous areas. This three basin river system presented a variety of areas colonized for *G. holbrooki* including marshlands, lakes, ponds and irrigation channels allocated at different distances, and thus represented our main point of interest to study local diversity levels and population structure of the species. In fact, some of the populations in these areas had been previously object of several ecological studies related with biological invasions (García-Berthou et al., 2000, 2005; Alcaraz and García-Berthou, 2007; Carmona-Catot et al., 2011).

2. Ebro River: It is the second largest river in the Iberian Peninsula with 928 km of length and 85.550 km^2 of drainage area. As we were interested in the effects of pollutants on population structure, samples were mainly collected in 2007 around the Flix reservoir, a small reservoir located 90 km upstream from the river mouth. Since 1949, a chemical plant manufacturing solvents and chlorinated pesticides deposited in Flix reservoir up to 3 x 10^5 tons of industrial waste contaminated with heavy metals, organochlorine pesticides and radionuclides, with well studied effects on local fauna (Navarro et al., 2009; Cid et al., 2010; Faria et al., 2010; Suarez-Serrano et al., 2010; Alcaraz et al., 2011; Carrasco et al.,

2011) including several fish species (Benejam et al., 2009) and humans (Montuori et al., 2006).

3. American samples: We selected samples from two American locations because they were suggested as close to the sources of the European invasions (Vidal et al., 2010; Sanz et al., 2013). This fish had been collected in 2005.

3.2   *DNA extractions and molecular analyses*

The use of DNA markers to amplify random genomic regions remains the most accessible and widely used method of elucidating the distribution of genetic variability within and among populations (DeFaveri et al., 2013). For this thesis we mainly extracted population genetics data from the samples by analyzing microsatellite loci variation. In addition, we studied pattens of variation in an allozyme locus (GPI) to study the effects of pollution on population structure.

1. Microsatellite loci: We used commercial kits to extract genomic DNA from a small piece of the caudal muscle of the fish. DNA was stored until performing the Polymerase Chain Reaction (PCR). Among all microsatellite loci published in literature for *G. holbrooki*, and its close relative species (*G. affinis*, *Poecilia reticulata*, *Poeciliopsis o. occidentalis*), we optimized two panels of five and six microsatellites that resulted polymorphic in our samples. Thus, each individual was genotyped for eleven microsatellite loci: Pooc-G$_{49}$, Mf13, Gafμ3, Gafμ5, Gafμ6, Gafμ7, Gaaf7, Gaaf9, Gaaf10, Gaaf13 and Gaaf15 (Parker et al., 1998; Zane et al., 1999; Spencer et al., 2000; Purcell et al., 2011). PCR conditions are specified in each chapter. In all of them, forward primers were fluorescently labeled, and genotype peaks were resolved on a 3130 Genetic Analyzer and using GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA).

2. Allozyme: Allelic variation at the glucosephosphate isomerase (GPI-2) locus has been observed in response to high concentrations of mercury in water (Congdon, 1994), exposure to arsenate (Newman et al., 1989), and thermal (Mulvey and Newman, 1995) and salinity (Congdon, 1994) stresses. To study genotypes in this locus, the head of each collected fish was homogenized in an Eppendorf tube with distilled water. Starch gel electrophoresis buffered with an amino-citrate was used to resolve gene products of the glucosephosphate isomerase enzyme. The locus was codominantly expressed and permitted direct counts of alleles from gel phenotypes.

3.3   *Data analyses*

There is a wide variety of data analyses that can be used on microsatellite and allozyme genotype data (reviewed in Selkoe and Toonen, 2006). In this thesis we used some of those that specifically allowed us to make inferences in population genetics of mosquitofish.

1. Genetic diversity and effective population size: To assess the levels of genetic diversity we estimated the observed heterozygosity (H$_O$), expected heterozygosity (H$_E$), the number of alleles per locus (A), and allele richness (A$_R$) either for the GPI-2 polymorphism and the microsatellite loci. The exact probability test of Guo and Thompson (1992)

implemented in GENEPOP 4 software (Rousset, 2008) was applied to check conformance of genotype frequencies to Hardy-Weinberg expectations (HWE) in all analyzed loci. MICROCHECKER (Van Oosterhout et al., 2004) was used to identify null alleles and their frequencies were estimated in FREENA (Chapuis and Estoup, 2007). We tested for recent population bottlenecks at the study sites using BOTTLENECK 1.2.02 (Piry et al., 1999). Effective population sizes (N_E) at each study location were estimated using linkage disequilibrium between loci in the LDNe 1.31 program (Waples and Do, 2008), or by using the online version of the ONeSAMP program (Tallmon et al., 2008).

2. Population differentiation and structure: Pairwise population differentiation (F_{ST}) and significance values were calculated using FSTAT software (Goudet, 2001). Isolation-by-distance (IBD) within and among watersheds was estimated from the correlation between pairwise genetic differentiation (linearized as $F_{ST}/1$-F_{ST}) and the log-transformed geographical distance (Rousset, 1997), with significance determined by Mantel tests using the IBD Web service 3.15 (Jensen et al., 2005). The minimum number of homogeneous units (K) over sampled individuals was estimated using the MCMC method in STRUCTURE 2.3.3 (Pritchard et al., 2000). The group-level Bayesian analysis in BAPS 5.4 (Corander et al., 2008) grouped populations that frequently exchanged individuals. Population genetic structure among populations was depicted by two-dimensional plots from the principal components analysis (PCA) of the allele frequencies matrix in GENALEX 6.4 (Peakall and Smouse, 2006), and by neighbor-joining (NJ) trees based on the D_A distance matrix (Nei et al., 1983) using POPULATIONS software (Langella, 2002). Major genetic discontinuities between locations were assessed using Monmonier algorithm in BARRIER 2.2 (Manni et al., 2004), which detects hidden barriers to gene flow among sites according to their geographical coordinates and relative genetic differentiation (F_{ST}). Analyses of molecular variance (AMOVA) were conducted in ARLEQUIN 3.5 (Excoffier and Lischer, 2010) in which several hierarchical models were tested for partitioning the genetic diversity into three levels: within locations, among locations within regions, and among regions.

3. Dispersal and gene flow: Contemporary migration rates among populations were estimated by using the Bayesian inference as implemented in BAYESASS 3.0 software (Wilson and Rannala, 2003), and connectivity among locations was analyzed using the assignment tests implemented in GENECLASS 2 (Piry et al., 2004).

4

Results

The main objectives of this thesis were addressed through the empirical analyses of genetic data obtained from introduced populations of *G. holbrooki* in Mediterranean streams. The results are presented and discussed in three different chapters structured as scientific articles:

1. In Chapter 1, entitled *Gene Flow and Maintenance of Genetic Diversity in Invasive Mosquitofish, Gambusia holbrooki*, we used microsatellite loci to determine population structure patterns among Mediterranean streams, to evaluate putative losses of genetic diversity due to introductions so we could understand the mechanisms that contribute towards retaining levels of diversity within populations inhabiting invaded rivers compared to populations in native basins. This article was originally published in PLoS ONE in December 16th 2013 as Díez-del Molino et al. (2013), but has been adapted to maintain the thesis format.

2. In Chapter 2, *Effects of Water Pollution on the Genetic Population Structure of Invasive Mosquitofish*, we analyzed mosquitofish genetic variation at several microsatellite loci and the GPI-2 locus on population along the Ebro River (Spain), upstream and downstream of the polluted sediments of the Flix reservoir. Our main aim was to test whether anthropogenic perturbations (pollution and dams) altered the genetic diversity and structure of introduced mosquitofish populations.

3. In the Chapter 3, entitled *Temporal Genetic Dynamics among Mosquitofish Gambusia holbrooki Populations in Invaded Watersheds*, we used microsatellite loci to analyze four different cohorts of *G. holbrooki* belonging to the same locations in order to characterize the role of temporal variation of gene flow patterns and population genetic dynamics in invasive populations.

Chapter 1

Gene Flow and Maintenance of Genetic Diversity in Invasive Mosquitofish (*Gambusia holbrooki*)

4.1   Gene Flow and Maintenance of Genetic Diversity in Invasive Mosquitofish (*Gambusia holbrooki*)

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Abstract

Genetic analyses contribute to studies of biological invasions by mapping the origin and dispersal patterns of invasive species occupying new territories. Using microsatellite loci, we assessed the genetic diversity and spatial population structure of mosquitofish *(Gambusia holbrooki)* that had invaded Spanish watersheds, along with the American locations close to the suspected potential source populations. Mosquitofish populations from the Spanish streams that were studied had similar levels of genetic diversity to the American samples; therefore, these populations did not appear to have undergone substantial losses of genetic diversity during the invasion process. Population structure analyses indicated that the Spanish populations fell into four main clusters, which were primarily associated with hydrography. Dispersal patterns indicated that local populations were highly connected upstream and downstream through active dispersal, with an average of 21.5% fish from other locations in each population. After initially introducing fish to one location in a given basin, such dispersal potential might contribute to the spread and colonization of suitable habitats throughout the entire river basin. The two-dimension isolation-by-distance pattern here obtained, indicated that the human-mediated translocation of mosquitofish among the three study basins is a regular occurrence. Overall, both phenomena, high natural dispersal and human translocation, favor gene flow among river basins and the retention of high genetic diversity, which might help retain the invasive potential of mosquitofish populations.

Keywords: evolutionary potential, gene flow, genetic diversity, biological invasions, isolation-by-distance, Poeciliidae.

4.1.1   Background

Biological invasions are a central component of global change, and a major threat to the biodiversity of freshwater ecosystems (Dudgeon et al., 2006). Invaders may change radically the functioning of an ecosystem, to which they are introduced, resulting in the decline or extinction of native species through predation, competition, and habitat alteration (Levine, 2008). Often, biological invasions begin when humans introduce a few individuals of a species to a new environment. Once established, the new population spreads to neighboring locations by natural dispersal. For example, the Eurasian spread of the topmouth gudgeon *(Pseudorasbora parva)* began accidentally when humans introduced it during the establishment of new cultured stocks of the common carp *(Cyprinus carpio)*, and was followed by further natural dispersal of short distances (Gozlan et al., 2010). Genetic variation is closely linked to the success of biological invasions (Lavergne and Molofsky, 2007). When introductions begin with just a few individuals, reduced genetic diversity is expected during the first stages of the invasion (Lockwood et al., 2005; Roman and Darling, 2007). However, several studies have shown high diversity in populations at later stages of the invasion process (see Dlugosch and Parker, 2008). The recovery of genetic diversity in invaded territories might result from

gene flow between recently established populations within the invaded range that have become increasingly interconnected. Multiple introductions from divergent stocks also contribute towards increasing local diversity in invaded territories (Kolbe et al., 2004), particularly if these introductions occur separately in time from the initial founder event (e.g. Keller and Taylor, 2010).

The eastern mosquitofish, *Gambusia holbrooki*, is one of the most commonly introduced freshwater species (Lowe et al., 2004). At present, established populations of this species in Europe, Africa, Asia, and Australia are causing local extinction and decline of several native fish and amphibian species (Alcaraz et al., 2008; Pyke et al., 2008). For example, the aggressive behavior of *G. holbrooki* has caused a decline in the feeding rates and reproductive success of two Iberian endemic fish species, *Valencia hispanica* and *Aphanius iberus* (Rincón et al., 2002). Similarly, (see Carmona-Catot et al., 2013) showed that introduced *G. holbrooki* were able to competitively displace *A. iberus* populations. Mosquitofish introductions were originally supported by governmental health agencies to control mosquito populations, which are vectors of various diseases, such as malaria (Meffe et al., 1989). In Europe, 12 individuals of *G. holbrook*i were initially introduced into a pond in southern Spain in 1921 (Vidal et al., 2010). Subsequently, humans spread *G. holbrooki* throughout the Mediterranean basin (García-Berthou et al., 2005). Despite their small size, mosquitofish are extremely successful in new environments (Meffe et al., 1989). Both the invertivorous diet and wide ecological tolerance of *G. holbrooki* have probably contributed to its successful integration into Iberian fish communities (Ribeiro et al., 2008).

Temporal fluctuations in population size reduce the average effective population size (N_E), with reduced effective sizes intensifying the loss of diversity due to genetic drift (Frankham, 1995). Although there is a major decline in the size of mosquitofish populations during winter after the summer flush (Krumholz, 1948), several studies have indicated high genetic diversity within American populations that often exceeds average values described for freshwater fishes (Hernandez-Martich and Smith, 1990). The high reproductive potential generated by overwintering pregnant females, multiple paternity, and offspring reaching maturity within a few weeks probably contribute towards maintaining large effective population sizes and preventing the loss of population diversity (Echelle et al., 1989; Zane et al., 1999; Spencer et al., 2000). Moreover, gene flow between seasonally isolated demes favors population diversity in large territories (Smith et al., 1983). For instance, sporadic individual exchange among close populations prevents divergence among collections within basins in invaded territories (Ayres et al., 2010).

Several models, such as isolated populations with no current migration and metapopulations of ephemeral populations connected by gene flow, may explain the population structure of organisms in linear river systems (e.g. Barson et al., 2009). Native mosquitofish populations usually represent single breeding units (McClenaghan et al., 1985), while large transects within a river basin are occupied by a single population with ephemeral local subpopulations (Smith et al., 1989). Source-sink dynamics are sometimes responsible for the population structure of mosquitofish (Smith et al., 1983; McElroy et al., 2011). Along a river system, dominant downstream gene flow increases the genetic diversity of lowland populations (Congdon, 1995; Hernandez-Martich and Smith, 1997)
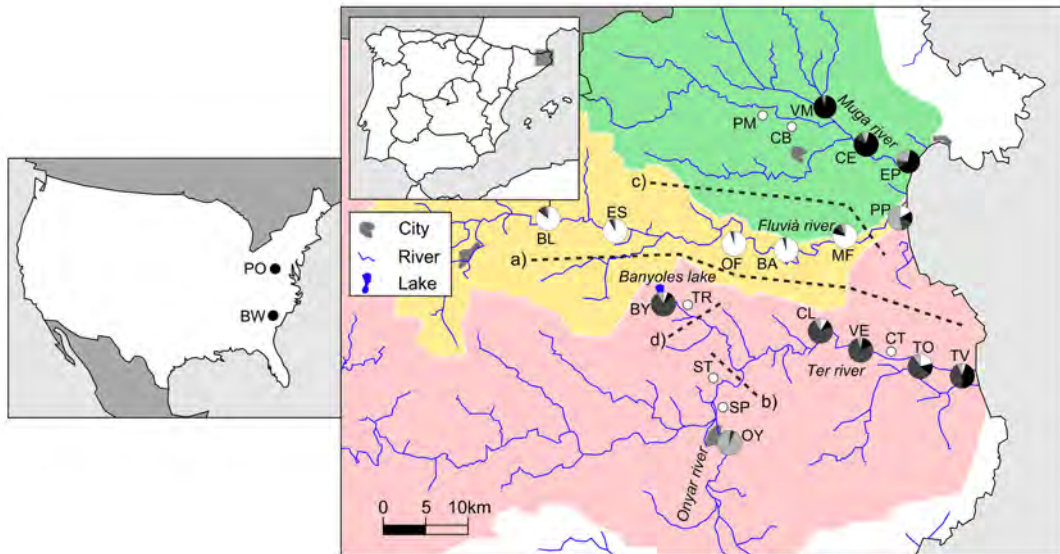
Figure 4.1: Geographical location of the collection sites. Sampled sites where *G. holbrooki* was not found are indicated with empty circles. Grey-scaled pie charts (white, light grey, dark grey, and black) represent mean proportional ancestry of every sampled site attributed to each cluster inferred by STRUCTURE. Watersheds are coloured. Dotted lines represent geographical barriers indicated by BARRIER and the letters indicate the order in which the program detected these barriers. Location codes are presented in Table 3.1. (A detailed map with information about the road network is available at: http://mapsengine. google.com/map/viewer?mid=zgd4mwb-ESLE.kSE9TUfF2uQ4)

DNA molecular markers contribute to improving our understanding of evolutionary changes that occur during biological invasions (Allendorf and Lundquist, 2003; Stepien et al., 2005; Frankham, 2010; Blanchet, 2012). Highly polymorphic microsatellite loci provide the discrimination required to address questions about population structure and gene flow (Spencer et al., 2000; Barson et al., 2009). In this study, we used microsatellite loci to evaluate putative losses of genetic variation during introductions of G. holbrooki. We aimed to understand the mechanisms that contribute towards retaining levels of diversity within populations inhabiting invaded rivers compared to populations in native basins.

4.1.2 Methods

Ethics statement

Animal samples were collected and manipulated under a permit (SF/012/2011) provided by the Agriculture, Fisheries, Food and Environment Department of the Autonomous Community of Catalonia. All work meets the requirements stated by the Spanish (RD53/2013) and Catalonian (D214/1997) laws of animal care, and experimentation.

Table 4.1: Description of the study locations. ND: *Gambusia holbrooki* not detected. Geographical coordinates: all longitudes are East, and latitudes North. N: sample size (females)

Basin	Location	Code	Coordinates	N
Muga	Pont de Molins	PM	2°57'11.49" 42°18'9.41"	ND
	Cabanes	CB	2°58'40.08", 42°17'55.68"	ND
	Vilanova de la Muga	VM	3°2'29.38", 42°16'49.86"	40 (20)
	Castello d'Empúries	CE	3°4'16.16", 42°15'17.54"	40 (20)
	Empuriabrava	EP	3°7'26.78", 42°14'14.97"	40 (20)
Fluvià	Besalú	BL	2°44'9.012", 42°11'27.41"	40 (27)
	Esponellà	ES	2°47'41.24", 42°11'0.268"	40 (20)
	Orfes	OF	2°52'12.54", 42°10'14.20"	40 (20)
	Báscara	BA	2°54'51.88", 42°9'49.76"	40 (20)
	Sant Miquel de Fluvià	MF	3°0'46.72", 42°9'56.04"	40 (20)
	Sant Pere Pescador	PP	3°4'18.02", 42°10'44.81"	27 (12)
Ter	Banyoles	BY	2°44'54.49", 42°7'7.317"	40 (20)
	Terri	TR	2°46'39.00', 42°7'1.704"	ND
	Onyar	OY	2°49'48.00", 41°58'25.53"	40 (22)
	Sant Ponç	SP	2°49'20.61", 41°59'33.67"	ND
	Sarrià de Ter	ST	2°49'33.37", 42°0'49.66"	ND
	Colomers	CL	2°59'8.999", 42°4'58.51"	40 (20)
	Verges	VE	3°2'38.79", 42°3'11.45"	11 (6)
	Canet de la Tallada	CT	3°4'5.232", 42°2'29.67"	ND
	Torroella de Montgrí	TO	3°9'7.177", 42°1'31.77"	40 (20)
	Ter Vell	TV	3°11'43.51", 42°2'42.84"	38 (34)
America	Potomac River	PO	38°38'60.0", 77°11'0.0"	36 (25)
	Brunswick	BW	34°16'60.0", 78°29'0.0"	16 (7)

Note: The 'Date' column of the published version of this table have been omitted with aesthetic purposes.

Sample collection

A total of 556 *G. holbrooki* were collected from 15 sites along three watersheds (Muga, Fluvià, and Ter rivers) in northeaster Spain. The largest of these rivers is the Ter, with a basin area of 2955 km^2, with its headwaters in the Pyrenees and its upper course being partially snow-fed. The Fluvià (974 km^2) and Muga (758 km^2) are typical Mediterranean streams with smaller watersheds, and have their headwaters located in mountainous areas. All three rivers are subject to a Mediterranean climate, with severe summer droughts and autumn floods (Trigo et al., 2004). The Ter and Muga rivers have many small weirs, along with a few large dams that form major barriers, altering connectivity among fish populations, whereas the Fluvià only has weirs. Mosquitofish are currently absent from the upper course of these watersheds; hence, we collected samples from the middle and lower courses of these three watersheds (Figure 4.1, Table 4.1). Sampling sites were shallow areas (<1.5 m depth) along the riverbank, with low water velocity and dense vegetation, usually reed beds (Phragmites australis). We also analyzed

36 individuals from the Potomac River (Washington) and 16 individuals from Brunswick (North Carolina), because it has been suggested that these populations are the closest to the main American source of *G. holbrooki* individuals that were introduced to Europe (Vidal et al., 2010).

Gambusia holbrooki specimens from Iberian rivers were collected from the riverbank using dip nets. All samples were collected from July to August 2010 and, to minimize any seasonal effects on population demography, only adult individuals born during the spring of the same year were selected by discarding females with a standard length of less than 2.5cm and more than 3.5cm, and males with a body length of less than 2.0cm (Carmona-Catot et al., 2011). Individuals were classified as adult males if a fully formed gonopodium was present, and as females if not. We attempted to collect 20 males and 20 females from each site; however, adult fish availability modified this ratio (Table 4.1). Whole fish were euthanized by lethal sedation in situ, and then preserved in 96% ethanol until DNA was extracted at the laboratory.

DNA extraction and microsatellite analyses

For each fish that was collected, genomic DNA was isolated from the caudal muscle using the Realpure Genomic DNA extraction toolkit (Durviz SL, Valencia, Spain) following the manufacturer's instructions. Genomic DNA was stored at -20°C until further use in Polymerase Chain Reactions (PCRs). Variation was analyzed at 11 loci (Pooc-G$_4$9, Mf13, and Gafμ3, Gafμ5, Gafμ6, Gafμ7, Gaaf7, Gaaf9, Gaaf10, Gaaf13, and Gaaf15), with two optimized multiplex PCR. Both multiplex PCR were conducted under the same conditions: 30 μl of reaction volume containing 5-15 ng genomic DNA, 0.34 μM of each primer, 200 μM dNTPs, 1.5 mM MgCl2, and 0.75 units of Taq polymerase. The PCR cycling conditions were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of 30 s at 94°C, 90 s at 60°C, 90 s at 72°C, and ending with a final extension of 10 min at 72°C. Forward primers were fluorescently labeled, and genotype peaks were resolved on a 3130 Genetic Analyzer and using GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA).

Genetic diversity within locations

Genetic diversity within each study site was estimated from direct counts as the mean observed heterozygosity (H$_O$) and the number of alleles per locus (A). Genetic diversity was also measured using the estimated expected heterozygosity (H$_E$) and allelic richness (A$_R$) from allele frequencies using FSTAT 2.9.3 (Goudet, 2001). Using GENEPOP 4.0 (Raymond and Rousset, 1995), we measured the Hardy-Weinberg equilibrium (HWE) at each site, and tested for linkage disequilibrium between all pairs of loci. We corrected for multiple comparisons using the sequential Bonferroni test (Rice, 1989). The presence of null alleles was detected using MICROCHECKER 2.2.3 (Van Oosterhout et al., 2004), and their frequencies were estimated in FREENA (Chapuis and Estoup, 2007). We tested for recent population bottlenecks at the study sites using BOTTLENECK 1.2.02 (Piry et al., 1999).

Genetic structure within and among rivers

Pairwise population differentiation (F$_{ST}$) and significance values were calculated

Table 4.2: Genetic diversity of *Gambusia holbrooki* in the study locations. Average number of alleles (A), allele richness (A_R), average observed heterozygosis (H_O), average expected heterozygosis (H_E), and fixation index (F_{IS}). * Significant Hardy-Weinberg disequilibria after Bonferroni correction (P = 0.05). Location codes are presented in Table 4.1

Basin	Location code	A	A_R	H_O	H_E	F_{IS}
Muga	VM	3.27	2.68	0.393	0.434	0.094*
	CE	3.63	3.16	0.414	0.5	0.173*
	EP	3.09	2.85	0.442	0.478	0.075*
Fluvià	BL	3	2.56	0.389	0.417	0.066
	ES	2.91	2.45	0.371	0.387	0.041*
	OF	3.18	2.53	0.336	0.358	0.061
	BA	3.09	2.51	0.35	0.345	-0.015
	MF	3.55	2.84	0.402	0.42	0.042*
	PP	3.73	3.14	0.475	0.489	0.031
Ter	BY	3.27	2.65	0.434	0.453	0.042
	OY	3.36	2.87	0.449	0.464	0.03
	CL	4	3.31	0.438	0.482	0.092*
	VE	2.91	2.85	0.413	0.411	-0.004
	TO	3.55	3.08	0.457	0.474	0.036*
	TV	3.82	3.03	0.404	0.46	0.121
America	PO	5.45	4.8	0.457	0.577	0.213*
	BW	4.27	4.27	0.472	0.537	0.149

using FSTAT software. To assess the relevance of stepwise mutations on population differentiation (R_{ST}), an allele permutation test was performed with 1000 randomizations that simulated the distribution of allele sizes and R_{ST} values using SPAGEDI version 1.1 (Hardy and Vekemans, 2002). Allele richness and gene diversity patterns (H_E and F_{ST}) within basins were compared among basins using permutation tests in FSTAT (1000 permutations). Non-parametric Wilcoxon signed-rank tests were used to compare allele richness and gene diversity (H_E) between upstream and downstream collections within each study basin.

Isolation-by-distance (IBD) within and among watersheds was estimated from the correlation between genetic and geographical distance matrices among sampling sites. We used geographical distances, rather than hydrographical distances; because natural dispersal within linear river basins or human-mediated translocation by road could be involved in the connectivity between locations, given their geographical proximity (less than 50 km on average) and anastomosed road network (Figure 4.1). The geographical distances between sample sites were estimated using Google Earth. Pairwise genetic differentiation was linearized as $F_{ST}/(1-F_{ST})$ and geographical distance was log-transformed for these analyses (Rousset, 1997). Significance was determined by Mantel tests with 10000 permutations using the IBD Web service 3.15 (Jensen et al., 2005). Additional information was obtained from the regression analyses of the estimates of the effective number of migrants (Nm) between populations pairs Nm = (1 - F_{ST})/4F_{ST} and their geographical distances (both variables log-transformed). Negative relationships

indicate IBD, and the slope (b) of the linear regression ($\log Nm = a + b \times \log d$, where d equals geographical distance) is -1 for one-dimensional stepping stones models and -0.5 for the two-dimensional models (Slatkin, 1993).

The minimum number of homogeneous units (K) over sampled individuals was estimated using the MCMC method in STRUCTURE 2.3.3 (Pritchard et al., 2000). Runs for each possible K (1 to 15) were repeated 10 times. Each run used a burn-in of 40000 iterations, a run length of 100000 iterations, and the model of independent allele frequencies. The most likely value of K was selected following Evanno et al. (2005). The group-level Bayesian analysis in BAPS 5.4 (Corander et al., 2008) grouped populations that frequently exchanged individuals. BAPS analyses were repeated 10 times, with the maximum number of clusters set to 15. While STRUCTURE results tend to be conservative in the number of clusters detected providing ancestral information related with the history of introductions of the species, BAPS performed better in clustering together populations with recent gene flow (e.g. Vidal et al., 2012). In addition, genetic differentiation among populations was depicted by two-dimensional plots from the principal components analysis (PCA) of the allele frequencies matrix in GENALEX 6.4 (Peakall and Smouse, 2006).

Major genetic discontinuities in the study area were assessed using Monmonier algorithm in BARRIER 2.2 (Manni et al., 2004), which detects hidden barriers to gene flow among sites according to their geographical coordinates and relative genetic differentiation (F_{ST}). These analyses were conducted using the F_{ST} matrices from single-locus information corrected by the presence of null alleles (FREENA software). We identified the main barriers for each locus, and only retained those confirmed by at least six loci.

Analyses of molecular variance (AMOVA) were conducted in ARLEQUIN 3.5 (Excoffier and Lischer, 2010). Two hierarchical models were tested for partitioning the genetic diversity into three levels: within locations, among locations within regions, and among regions. The first AMOVA model assumed a pure hydrographical pattern of population hierarchy (watersheds = regions). Another AMOVA grouped locations according to the main clusters identified by STRUCTURE (clusters = regions).

Gene flow

Contemporary migration rates among populations were estimated by using the Bayesian inference as implemented in BAYESASS 3.0 software (Wilson and Rannala, 2003), which is a method that does not assume migration-drift or Hardy-Weinberg equilibrium. A total of 5×10^6 iterations were performed until the MCMC chains reached stationarity (i.e., constant over time). Migration parameters were estimated by sampling every 1000 iterations after a burn-in of 10^6 iterations. Delta values were adjusted following the BAYESASS manual recommendations. Five runs using different starting points were performed, and the results with the highest likelihood were retained.

4.1.3 **Results**

Diversity within locations

At the invaded locations, all 11 microsatellite loci were polymorphic, ranging in

variability from just two alleles (Mf13, Gaaf15, and Gaaf9 loci) to nine (Gaaf13 locus). Average allelic richness (A_R) ranged from 2.45 in ES (Fluvià River) to 3.31 in CO (Ter River) (Table 2). The observed heterozygosity (H_O) ranged from 0.336 in OF (Fluvià River) to 0.475 in PP (Fluvià River), and the expected heterozygosity (H_E) ranged from 0.345 in BA (Fluvià River) to 0.500 in CE (Muga River). At the studied American collections, diversity levels averaged 4.45 for allelic richness, 0.465 for H_O, and 0.557 for H_E. After adjusting for differences in sample sizes, FSTAT permutation tests demonstrated lower allele richness (P = 0.009) and H_E (P = 0.010) in the invaded Spanish locations compared to the potential American sources. Non-significant F_{ST} differentiation was detected between males and females (Table 4.3). Subsequent analyses were then performed pooling both sexes as a single collection for each location.

Deviations from HWE were detected at seven Spanish locations after Bonferroni correction (Table 4.2). According to MICROCHECKER, null alleles were responsible for the observed positive F_{IS} values. Significant null allele frequency was estimated at Gafμ5 (q = 0.071 in ES, Fluvià River; q = 0.089 in TO, Ter River), Gafμ6 (q = 0.149 in ES and q = 0.231 in CE, Muga River), Gafμ7 (q = 0.172 in MF, Fluvià River), Gaaf10 (q = 0.081 in CE, Muga River; q = 0.147 in CO, q = 0.126 in TO and q = 0.132 in TV, Ter River), and Gaaf15 (q = 0.231 in VM and q = 0.265 in CE, Muga River). No significant pairwise linkage disequilibria were observed. According to the BOTTLENECK analyses, heterozygosity excess relative to mutation-drift equilibrium was observed at CE (Muga River), BL, and MF (Fluvià River), and VE, and OY (Ter River); however, the allele-shift model test reported additional signals for a bottleneck at VE only.

Population divergence within and among basins

No significant differences between F_{ST} and R_{ST} estimates were observed (P = 0.345), indicating that local mutations have limited effects on population structure. Significant genetic differentiation was detected between almost all population pairs (Table 4.3), except for two neighboring sites in the Muga (VM and CE locations) and Fluvià (OF and BA locations) rivers (Table 4.3). Estimated average genetic differentiation among invaded Spanish locations was F_{ST} = 0.1641, with no substantial change in this estimate after correcting for null alleles (F_{ST} = 0.1642). The collection OY from the Onyar tributary in the Ter basin had the largest average pairwise F_{ST} (0.194).

Despite the different values on estimates of average allele richness, the average expected heterozygosity, and population differentiation observed in the Iberian rivers (Table 4.4), FSTAT permutation tests only indicated marginally reduced heterozygosity in the Fluvià basin compared to the Muga (P = 0.062) and Ter basins (P = 0.052). Overall, among-basin differences in genetic diversity (allele richness and heterozygosity) were non-significant between upstream and downstream locations. Marginal (P = 0.067) increased divergence among the upstream locations of the three basins was indicated (Table 4.4). Within-basin comparisons with Wilcoxon signed-rank tests indicated higher allele richness for just the downstream location of the Fluvià River (P = 0.042).

Table 4.3: Genetic (F$_{ST}$ corrected by the presence of null alleles, below the diagonal) and geographical distances (km, above the diagonal) between samples.

| Basin | Code | Muga | | | Fluvià | | | | | | Ter | | | | | |
		VM	CE	EP	BL	ES	OF	BA	MF	PP	OY	BY	CL	VE	TO	TV
Muga	VM	**-0.011**	3.67	7.14	29.01	22.87	18.06	16.9	12.78	11.79	29.05	38.4	22.45	24.65	27.94	30.06
	CE	**0.02**	**-0.006**	3.85	31.11	23.96	18.59	17.22	11.65	7.94	29.62	37.24	20.43	22.31	25.01	26.84
	EP	0.06	0.056	**-0.001**	33.85	26.71	21.12	19.01	12.72	6.5	32.16	37.88	20.6	21.61	23.1	24.65
Fluvià	BL	0.303	0.268	0.24	**0.028**	8.28	14.01	17.5	24.07	31.45	8.98	28.26	26.39	32.13	38.97	43
	ES	0.293	0.251	0.225	0.037	**0.026**	5.93	9.18	16.07	23.58	6.72	23.83	18.55	24.32	30.97	35.03
	OF	0.32	0.276	0.283	0.094	0.052	**0.01**	3.5	9.96	17.52	11.02	23.29	13.58	19.34	25.68	29.55
	BA	0.313	0.273	0.262	0.122	0.044	**0.021**	**-0.002**	6.93	14.83	13.43	22.34	10.69	16.08	22.51	26.4
	MF	0.221	0.178	0.166	0.131	0.06	0.056	0.043	**0.028**	7.85	20.17	25.57	9.49	13.33	18.21	21.53
	PP	0.146	0.114	0.046	0.195	0.177	0.239	0.22	0.129	**-0.007**	28.07	31.29	14.17	15.02	17.06	19.32
Ter	OY	0.233	0.196	0.124	0.271	0.254	0.337	0.32	0.24	0.09	**0.006**	19.13	17.6	19.32	24.42	27.57
	BY	0.166	0.13	0.125	0.14	0.151	0.184	0.2	0.149	0.126	0.197	**-0.001**	19.36	25.05	31.75	39.96
	CL	0.151	0.107	0.101	0.181	0.146	0.172	0.168	0.097	0.094	0.175	0.061	**0.011**	5.9	12.66	16.43
	VE	0.187	0.142	0.14	0.226	0.194	0.242	0.231	0.146	0.12	0.21	0.108	0.041	**-0.046**	7	10.99
	TO	0.135	0.108	0.084	0.153	0.133	0.166	0.158	0.093	0.091	0.176	0.047	0.032	0.064	**0.003**	4.18
	TV	0.108	0.08	0.064	0.244	0.221	0.245	0.234	0.143	0.124	0.204	0.108	0.057	0.105	0.065	**0.066**

Non-significant differences were obtained in the Muga River basin, and an unexpected higher richness was indicated at the upstream CL location compared to the downstream TV in the Ter River basin (P = 0.034). None of these changes in allele richness between upstream and downstream locations resulted in significant differences on the estimated amount of heterozygosity.

Table 4.4: Genetic diversity patterns within and among the studied locations. Values of average allele richness (A_R), expected heterozygosis (H_E), and population differentiation (F_{ST}) are shown. Location codes are presented in Table 4.1.

Region / basin	A_R	H_E	F_{ST}
American sources	3.87	0.552	0.242
Spain (all study locations)	2.83	0.432	0.164
Muga River	2.89	0.470	0.042
Fluvià River	2.66	0.397	0.104
Ter River	2.97	0.463	0.116
Upstream (VM, BL, CL locations)	2.85	0.443	0.215
Downstream (EP, PP, TV locations)	3.00	0.473	0.073

Genetic and geographical distance matrices were positively correlated across all three watersheds (r = 0.326, P < 0.001). Within watersheds, only the Fluvià River displayed a significant correlation (Fluvià River, r = 0.694, P < 0.01). Significant negative regression of the log-transformed effective number of migrants and geographical distances were detected for the whole data set of the studied locations in Spain. Furthermore, as the slope of the regression (b = -0.629) was closer to -0.5 than to -1, a two-dimensional stepping stone model better explained the population relationships (Figure 4.2). Within watersheds, regression analyses of the number of migrants and distances were only significant for the Fluvià River, where a slope of -1.101 supported a one-dimensional stepping stone model. Marginal significance (P = 0.052) was obtained for the whole Ter River basin (b = -0.455); however, this significance disappeared when the analysis focused on the mainstem of the river (CL, VE, TO, and TV locations).

Although the number of clusters identified varied among analyses, the clusters basically reflected population relationships according to the extent of drainage connection. Evanno's method indicated four STRUCTURE clusters (Figure 4.4a). Cluster 1 grouped individuals from all sites in the Muga river basin, while cluster 2 grouped all samples from the Fluvià River watershed, except for PP. The third cluster grouped all samples from the Ter River populations, except the Onyar tributary, which was assigned to cluster 4. The coastal locations of EP, PP, and TV showed a remarkable degree of cluster admixture. The Monmonier's algorithm of BARRIER identified four barriers supported by at least six loci (Figure 4.1). The first (a) and third (c) barriers reflected the isolation of the three watersheds, with the exception of the PP location in the Fluvià River basin, which was grouped with the Muga River collections. The second (b) and fourth (d) barriers reflected the distinct genetic composition of the OY and BY locations separated from the mainstem of the Ter River by river transects where mosquitofish were not detected during our surveys. Overall, these results mainly agreed with the population relationships depicted by the two principal axes of the PCA analysis (Figure 4.3). The first
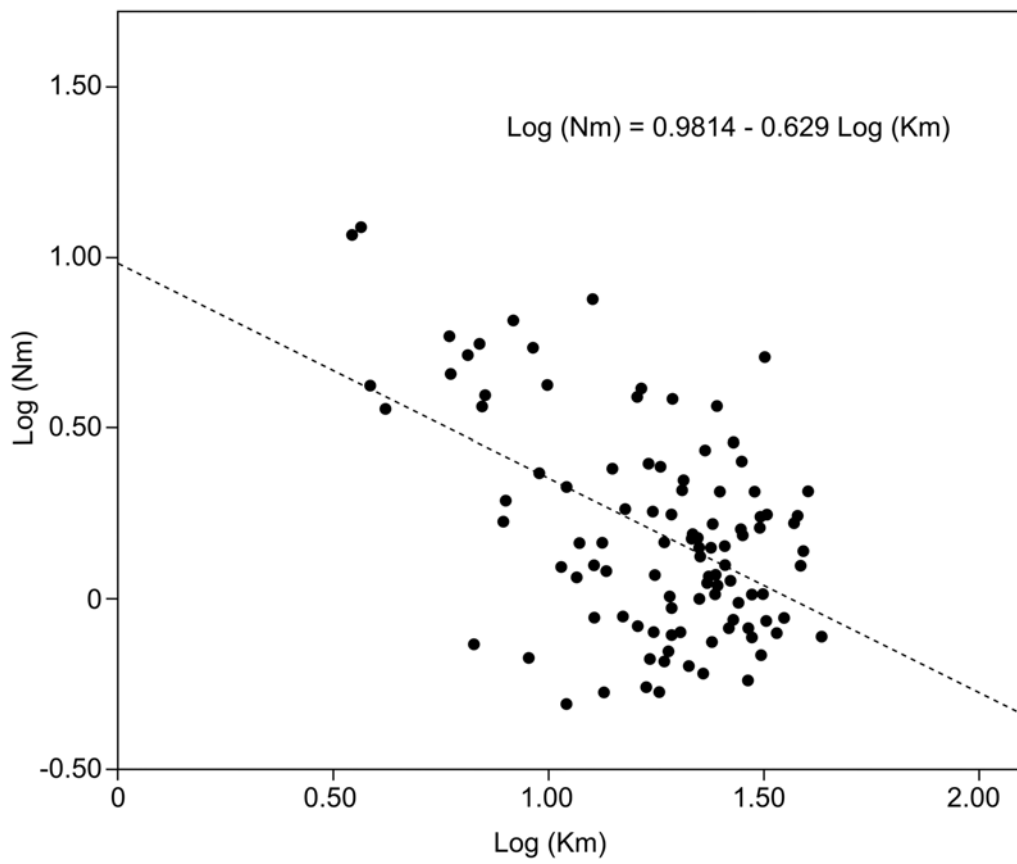
Figure 4.2: Linear regression of estimates of the effective number of migrants (Nm) and geographical distances between population pairs (both variables log-transformed, see Methods)

axis explained 52.9% of the allelic variance, and clearly differentiated the Fluvià River collections from the rest, with the exception of PP. The second axis (17.1%) separated the Muga and Ter River basins from the singular population of OY. BAPS identified 10 homogenous units within the study region, basically indicating that each collection represented a single panmictic group (Figure 4.4b). Only intra-basin locations that had the largest estimates of current gene flow (Table 4.5) were grouped together; specifically, VM and CE in the Muga basin, BL and ES and OF and BA in the Fluvià basin, and CL, VE, and TO in the Ter basin.

Hierarchical AMOVAs revealed that the genetic variance was significant at all levels, with most of the variance being attributed to individuals within locations (80.9-82.9%). In the hydrographical model, the variance assigned to divergence among populations within basins (8.8%) was smaller compared to the variance among river basins (10.3%). This pattern reflected the above noted population divergence among and within drainages. In the AMOVA based on the four STRUCTURE clusters, the proportion of genetic variance explained within clusters decreased to 6.8%, while the variance among these clusters increased (12.3%), probably reflecting the distinctiveness of the OY collection from locations in the mainstem of the Ter River.
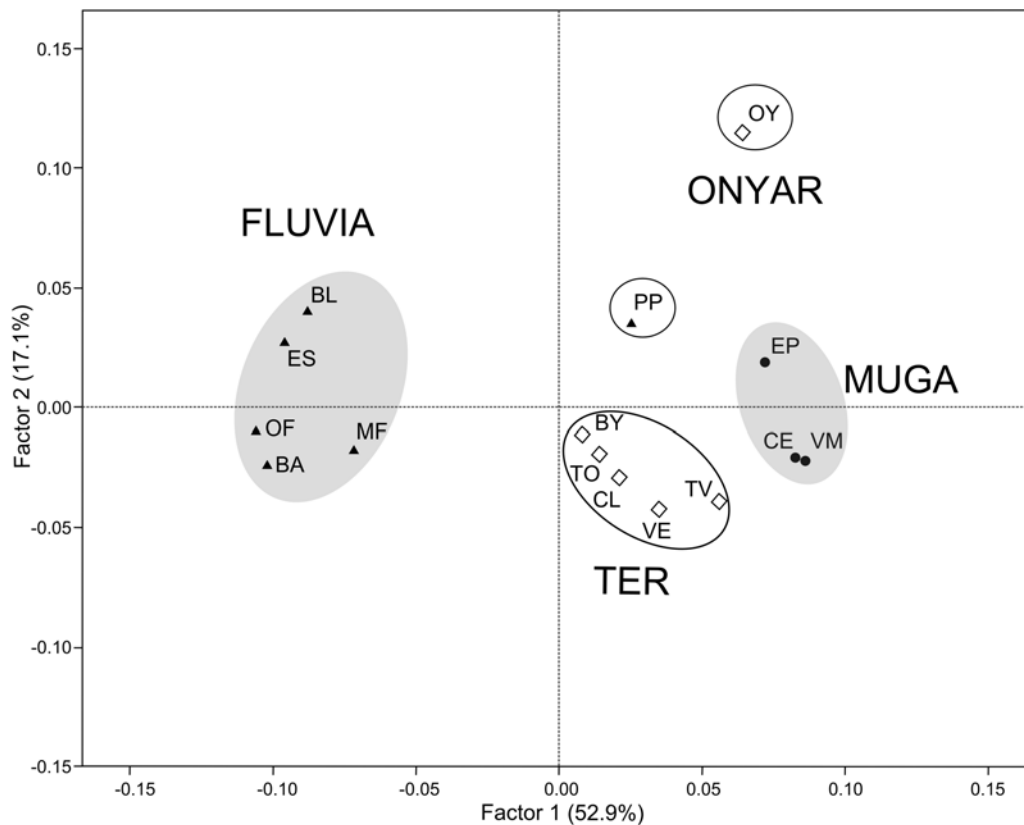
Figure 4.3: Principal component analysis (PCA) showing the relationships among the studied *G. holbrooki* populations. Samples are projected onto the plane formed by the first two principal axes. The first factor explained the 52.9% of total variance, the second 17.1%, and the third 13.5%. Empty circles indicate positive values of the third axis, while grey circles indicate negative values. Location codes are presented in Table 4.1

Overall, contemporary dispersal rates indicated an average of 21.4% of immigrant individuals at each location (range 11-31%, Table 5). As a conservative rule, we only discussed the 5% of highest estimates (11 out of 210 values). Within this framework, the most significant estimates of dispersal rates were basically downstream within rivers. Only at the ES site, and particularly at the OF site, a significant proportion of individuals were upstream immigrants from BA. BAYESASS also indicated the presence of gene flow among river basins, particularly from EP (Muga River) to PP (Fluvià River), and from the Onyar River (OY, Ter basin) to PP (Fluvià River).

4.1.4   *Discussion*

Genetic diversity and invasive potential retained in Spanish basins

In colonized territories, the level of genetic diversity of the invading species is expected to be reduced compared to original sources as a result of founder events (Allendorf and Lundquist, 2003; Lavergne and Molofsky, 2007; Dlugosch and Parker, 2008; Suarez and Tsutsui, 2008). This phenomenon has been suggested for European mosquitofish populations when compared against the American collections from Florida

(Grapputo et al., 2006). For a more accurate evaluation of the effect of founder events on the genetic diversity and evolutionary potential of the Spanish populations, we compared the level of genetic diversity in the Spanish populations against those observed in the American populations considered to be the potential sources of the fish that were introduced to Europe. Previous studies have shown that the haplotype Hol1 is almost fixed in the Spanish collections and the American populations of Brunswick and Potomac River (Vidal et al., 2010). In addition, the Potomac River collection was the most closely related to European mosquitofish in a survey based on six microsatellites (Sanz et al., 2013). Genetic diversity declines by a factor of $(1-1/2N_E)$ per generation during a founder effect depending on the effective number (N_E) of introduced individuals (see for instance Lavergne and Molofsky, 2007). Historical records indicate that just 12 individuals of mosquitofish were introduced to Spain (Nájera, 1944). If we consider the best case scenario for diversity retention involving just a single generation founder effect with an effective population size of 12 individuals, the population should have preserved around 95% of the original genetic diversity, or even more if some of the specimens were gravid females, because multiple paternity increases the effective size in mosquitofish populations (Zane et al., 1999). With no relevant effects of mutations on population structure ($F_{ST} = R_{ST}$), all of the diversity present in the invaded range should be attributed to the population sources of the invasion. Based on the average diversity in the two American collections studied here ($H_E = 0.522$, Table 4.4), the estimated diversity at these source locations agreed with the estimated total diversity at the Spanish study region ($H_T = 0.522$). This observation indicates minimal, if any, loss of genetic diversity in the introduced populations of the Iberian Peninsula. Nevertheless, a significant reduction in allele richness was detected at the invaded Spanish locations, because this parameter is more sensitive to bottlenecks compared to average heterozygosity (Leberg, 1992; Spencer et al., 2000). It is therefore likely that introduced Spanish populations have not substantially reduced their evolutionary potential compared to American sources, because the levels of additive variance might still be less sensitive to bottlenecks compared to neutral variation (see reviews in Hänfling and Brandl, 1998; Blanchet, 2012). For example, introduced Australian populations of the guppy (*Poecilia reticulata*) showed strong genetic bottlenecks in genetic diversity when measured with neutral markers; yet, these populations retained substantial additive variation (Lindholm et al., 2005).

Lower neutral genetic variation is often detected in populations at the limit of the distribution range (Eckert et al., 2008). Reduced diversity and singular mtDNA haplotypes of *G. holbrooki* in northern American drainages indicated postglacial colonization from refuge populations in Georgia or Florida (Scribner and Avise, 1993). A recent work based on microsatellite variation confirmed an important reduction in allele richness (up to 50%) and heterozygosity (up to 30%) of American *G. holbrooki* populations in North Carolina and northward compared to populations that occurred to the south in South Carolina and Florida (Sanz et al., 2013). Nevertheless, peripheral populations often display greater stress-adaptation favoring subsistence in unstable environments (Hardie and Hutchings, 2010). Hence, available information about species with broad distributions indicates that less-stable habitats within native ranges serve as frequent sources of invasive populations (see Lee and Gelembiuk, 2008). If this was the case for mosquitofish, marginal populations of the northward range of America might

have already acquired the evolutionary changes to be invasive during the postglacial period, as far as substantial additive variation could be retained during related founder effects despite losses of neutral genetic variation. Therefore, the American mosquitofish sources used in the European introduction might represent an *invasive bridgehead*. As defined by Lombaert et al. (2010), invasive bridgeheads are particularly successful invasive populations that serve as the source of colonists for remote new territories. Because genetic diversity in the Spanish populations was not significantly reduced during the introduction, enough additive variance to respond to novel selection pressures in these non-native environments was probably conserved, favouring the successful and quick expansion of the species throughout the entire Mediterranean basin documented in historical records (Krumholz, 1948).
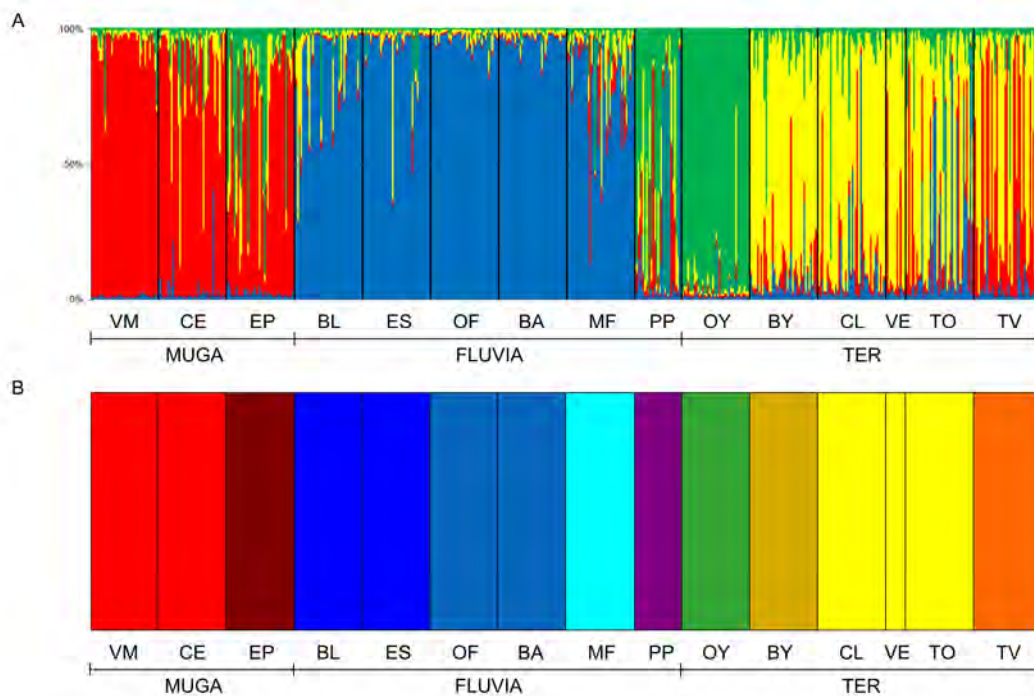
Figure 4.4: Bayesian analyses of population structure. Analyses were carried out with (a) STRUCTURE and (b) BAPS in the Iberian *G. holbrooki* populations. In (a) each individual is represented as a vertical bar partitioned into segments of different color according to the proportion of the genome belonging to each of the four identified clusters (K = 4). In (b) each location shows a different color according to the cluster to which it belongs. Location codes are presented in Table 4.1.

Genetic diversity and invasive potential retained in Spanish basins

Precise historical records are not available about the introduction of mosquitofish to the study basins. While *G. holbrooki* was first introduced to southwestern Spain in 1921, it was absent from the study basins in 1942 when insecticides (DDT) replaced mosquitofish as the major agent against malaria vectors. Malaria was eradicated in 1964 from Spain; however, mosquito control, including mosquitofish introductions, continued (reviewed in Fernández-Astasio, 2002). Mosquitofish were apparently introduced to Lake Banyoles

between 1952 and 1964, after they had already become established in other parts of the study watersheds (García-Berthou et al., 2000). Given that mosquitoes are abundant in marshlands dominating the lowlands of the three river basins (Boix et al., 2008), mosquitofish were probably first introduced into these lowland areas. The mosquitofish in the study river basins probably originated from well-established populations in central and southern Catalonia, such as the deltas of Llobregat and Ebro rivers, where mosquitofish were already present by 1942 (Fernández-Astasio, 2002). In the Ebro River, which is located around 300 km south of our study area, mosquitofish populations exhibit similar levels of total diversity ($H_T = 0.532$), with this diversity mainly being distributed within locations ($H_E = 0.523$)(Chapter 2).

Significant genetic divergence among study locations indicated the isolation of current mosquitofish populations both within and among the three studied basins. According to Smith et al. (1989), American mosquitofish populations along a river basin displayed a pattern of population divergence resulting from genetic drift and gene flow. However, some complex microgeographic patterns were also present as a result of interactions between dramatic demographic fluctuations and breeding structures complicated by multiple insemination and differential sex and cohort dispersal ability (Kennedy et al., 1986). Larger divergence among mosquitofish populations located in the upper reaches of the study basins might be related to founder events during dispersal along the basins, because the contribution of mutations to population structure was not significant. The average level of population diversity at these locations represented 85% of the total genetic diversity in the area. At each location, the stated percentage indicated 4-5 generations of bottlenecks from just 12 individuals in magnitude ($1-1/2N_E$ per generation of diversity losses), or more bottleneck-generations with larger N_E. In fact, signals of recent bottlenecks were detected at 30% of the locations, and affected populations from all river basins.

In the lowlands, differentiation among *G. holbrooki* populations exhibited similar patterns to that observed for the endemic killifish *Aphanius iberus*, in which increased gene flow was observed between populations during floods (García-Marín et al., 1991; Araguas et al., 2007). If flooding also connects mosquitofish populations, this process alone justified why barriers were not detected between the downstream mosquitofish populations in the Muga and Fluvià rivers, because the mouths of both rivers flow out of the same marshland (Aiguamolls de l'Empordà). In addition, substantial reductions in the population size of mosquitofish have been reported to recover within a few months after flooding (Chapman and Warburton, 2006). Moreover, pregnant females might buffer associated genetic bottlenecks (Nájera, 1944; Spencer et al., 2000). In the basins studies here, larger population divergence ($F_{ST} = 0.46$) has been detected among remaining native populations of the three-spine stickleback (*Gasterosteus aculeatus*) (Araguas et al., 2012). While stickleback remains in unpolluted streams with abundant aquatic vegetation (Clavero et al., 2009), mosquitofish are successful invaders of modified and disturbed habitats, such as ponds, irrigation ditches, and modified stream channels in urban areas (Meffe et al., 1989); such habitats allow increased gene flow among locations in invaded basins (e.g., Chapter 2).

Table 4.5: BAYESASS estimated migration rates among locations. Diagonal values (in italics): Proportions of non-migrant mosquitofish. The most relevant migration rates are shown in bold (see Results for further explanation).

To:	From: VM	CE	EP	BL	ES	OF	BA	MF	PP	CL	VE	TO	TV	BY	OY
VM	*0.8895*	0.0102	0.0173	0.0057	0.0061	0.0055	0.0057	0.0058	0.0076	0.0067	0.0066	0.0078	0.0088	0.0074	0.0092
CE	**0.1636**	*0.7131*	0.0119	0.0066	0.0079	0.0067	0.0083	0.0097	0.0069	0.0097	0.0069	0.0096	0.0218	0.0086	0.0086
EP	0.0194	0.0182	*0.8618*	0.0061	0.0064	0.0061	0.0059	0.0063	0.0096	0.0077	0.0059	0.0088	0.0212	0.0071	0.0094
BL	0.0061	0.0061	0.0065	*0.8848*	0.0127	0.0109	0.0094	0.0076	0.0073	0.0077	0.006	0.0073	0.006	0.0151	0.0065
ES	0.0063	0.0064	0.0065	**0.1882**	*0.6862*	0.009	**0.0423**	0.009	0.0062	0.0075	0.0062	0.007	0.0065	0.0064	0.0062
OF	0.0062	0.0062	0.006	0.0131	0.0298	*0.6898*	**0.1932**	0.0102	0.0062	0.0068	0.0064	0.0066	0.0061	0.0073	0.0062
BA	0.0061	0.0057	0.006	0.0216	**0.0611**	0.021	*0.8234*	0.01	0.006	0.0067	0.0054	0.0075	0.0064	0.0069	0.0063
MF	0.0079	0.0077	0.008	0.025	**0.1091**	0.019	**0.0509**	*0.7184*	0.0074	0.0085	0.0061	0.0091	0.0084	0.0074	0.007
PP	0.0095	0.011	**0.0634**	0.0112	0.0269	0.0094	0.0114	0.0179	*0.6908*	0.021	0.0087	0.0288	0.0201	0.0123	**0.0574**
CL	0.0103	0.0201	0.0166	0.0096	0.0136	0.0085	0.0082	0.0116	0.0081	*0.806*	0.0073	0.0225	0.0214	0.0268	0.0095
VE	0.0175	0.0142	0.0134	0.0136	0.0139	0.0133	0.0132	0.0133	0.0135	**0.1247**	*0.6804*	0.0244	0.0148	0.0173	0.0126
TO	0.0108	0.0084	0.0296	0.0251	0.0289	0.0086	0.0129	0.0093	0.0085	**0.0714**	0.0066	*0.7393*	0.0133	0.0191	0.0084
TV	0.0161	0.0253	0.0171	0.0064	0.0078	0.0061	0.0073	0.0077	0.0078	0.0324	0.0069	0.0177	*0.826*	0.0081	0.0075
BY	0.0073	0.0068	0.0072	0.0072	0.0069	0.0073	0.0062	0.0061	0.008	0.0087	0.0063	0.0119	0.0097	*0.8935*	0.0067
OY	0.0062	0.0067	0.0104	0.0062	0.0076	0.0076	0.0068	0.0077	0.0078	0.0073	0.0059	0.0082	0.007	0.0113	*0.8933*

BAYESASS indicated current relevant migration rates between some neighboring population pairs in all of the study basins. This phenomenon resulted in BAPS clustering the VM and CE locations in the Muga River, the BL and ES and the BA and OF in the Fluvià River basin, and the CL and VE in the Ter river basin. These location pairs were separated by a distance of 5.3 km on average (range 3.50-8.28), with significant dispersal occurring both downstream and upstream (BA to OF). High positive spatial autocorrelation of allele frequencies at hydrological distances of 6-150 km has been observed in American drainages (reviewed in Smith et al., 1989), indicating gene flow between distant locations within short time scales (few generations). In (Alemadi and Jenkins, 2007), the authors suggested that mosquitofish may disperse at rates greater than 800 m/day in unimpeded corridors. These observations indicate that the dispersal ability of mosquitofish is sufficient to colonize an entire basin from a single founder effect. Once a single population is established, further active upstream or passive downstream dispersal leads to the founding of new populations, and maintains high enough gene flow to preserve existing genetic diversity throughout all locations along the invaded river basin, and to overcome founder effects.

In the Spanish basins, isolation by distance was indicated among mosquitofish populations through the significant correlation between genetic and geographical distances in the whole territory. The significant negative relationship (b = -0.629) observed between the log-transformed effective number of migrants (Nm) and the geographical distances also supported a two-dimensional stepping stone model of gene flow. Thus, in addition to active linear and flood-mediated dispersal along river basins, human-mediated translocations between road-neighbouring populations from separate basins have probably contributed to the spread of mosquitofish in the area (for instance to OY and BY in the Ter river basin). In Australia, unreported and unregulated human-mediated dispersal has led to the introduction of mosquitofish to areas outside of its first sites of introduction (Ayres et al., 2010). In addition, a higher incidence of aquarists in more densely human populated areas (such as Girona in this study) might contribute to mosquitofish dispersal, through aquaria fish being discarded into urban ponds and river streams. Such practices have contributed to the dispersal of alien poeciliid species in Australia (Lintermans, 2004) and Spain (Ribeiro et al., 2008). Human-mediated translocations of endangered native species, such as *A. iberus* and *G. aculeatus*, are forbidden by Spanish laws directed towards protecting biodiversity. However, human-mediated dispersal might represent a major means of promoting gene flow between distant populations of mosquitofish, with such dispersal probably contributing towards retaining the high levels of genetic diversity within the populations of this species throughout the whole territory. It is also likely that human-mediated dispersal plays an important role in the maintenance of the invasive potential of these introduced populations, enabling them to outcompete the native fish.

Chapter 2

Effects of Water Pollution on the Genetic Population Structure of Invasive Mosquitofish

Unpublished manuscript

4.2   Effects of Water Pollution on the Genetic Population Structure of Invasive Mosquitofish

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Abstract

We analyzed variation at the GPI-2 locus and neutral microsatellite loci of eastern mosquitofish, *Gambusia holbrooki*, populations introduced to the Ebro River (Spain) above and below a dam (Flix reservoir), where severe chronic pollution has been well documented, to test whether the presence of a dam and high amounts of contaminants can alter the genetic diversity and population structure. Allele frequency changes at the GPI-2 locus of the nearest sites to the polluted sediments agree with previous results from studies in mercury-exposed populations of mosquitofish with an overall significant reduction in frequency of the GPI-2^{100} allele in polluted areas. Population structure results clearly showed the genetic distinction of the mosquitofish collected close to the polluted sediments both at the GPI-2 locus and the presumptive neutral microsatellite loci. Recent migration rates estimated from microsatellites suggested that around 30% of fish collected in every location were immigrants from upstream and downstream sources. High migration rates suggest that the effects of pollutants on mosquitofish genetic structure might be higher than observed and are compensated by immigration from other sites and explain the invasive success of mosquitofish.

Keywords: Heavy metals pollution, mercury, genetic population structure, river fragmentation, *Gambusia holbrooki*, invasive species.

4.2.1   Background

Widespread invaders in freshwater ecosystems such as the common carp *Cyprinus carpio*, the tilapias (*Oreochromis spp.*), or the mosquitofishes (*Gambusia affinis* and *G. holbrooki*) are often species tolerant to water pollution and habitat degradation (García-Berthou et al., 2005). The eastern mosquitofish *Gambusia holbrooki* is a tolerant, life-bearing fish native to part of North America that has been introduced in marshlands, lagoons and reservoirs worldwide and is considered as one of the worst invasive fishes (Pyke, 2005; Alcaraz et al., 2008). Mosquitofish populations often boom during the reproductive season in spring and summer but decline strongly during winter (Krumholz, 1948). Although these fluctuations in population size may reduce population genetic diversity, the huge reproductive potential involving maturity and reproduction in a few weeks and the capacity for sperm storage by females maintain large effective population sizes, and prevent losses on population diversity (Zane et al., 1999). Allozyme studies indicate high genetic diversity within mosquitofish populations, often exceeding average values described for freshwater fish species (Hernandez-Martich and Smith, 1990). Moreover, the gene flow between seasonally isolated demes favors population diversity in this species (Smith et al., 1983). For instance, sporadic individual exchange among close populations prevents divergence among collections within basins in invaded territories of Australia and Europe (Ayres et al., 2010; Díez-del Molino et al., 2013).

Chapter 3

Temporal Genetic Dynamics among Mosquitofish (*Gambusia holbrooki*) Populations in Invaded Watersheds

Unpublished manuscript

4.3 Temporal Genetic Dynamics among Mosquitofish (*Gambusia holbrooki*) Populations in Invaded Watersheds

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Abstract

The temporal components of genetic diversity and geographical structure of invasive mosquitofish populations are poorly known. Using microsatellite genotype data, we analyzed four consecutive cohorts of *G. holbrooki* from three different river basins aiming to determine temporal patterns of genetic variation and dispersal rates. Despite showing evidence of strong population size fluctuations, genetic diversity levels were maintained among local cohorts. We only detected allele frequency changes associated with seasonal flooding. Downstream gene flow coupled with increased connectivity at lowland locations may increase genetic diversity levels in these areas. A large proportion of local fish (up to 50%) were originated from locations within the same river basin. High dispersal capability, ecological tolerance, and reproductive traits likely promote upstream colonization. Finally, our results support the role of human-assisted translocations in the promotion of within -and among - basin gene flow and so can be considered a critical factor in both the maintenance of genetic diversity and the invasive spread towards upstream locations.

Keywords: Genetic diversity, temporal variation, population structure, river fragmentation, *Gambusia holbrooki*, invasive species.

4.3.1 Background

Poeciliids are a small, live-bearing fish species from tropical and temperate American continental waters that have high ecological tolerance and that have successfully colonized new territories worldwide (Meffe et al., 1995). Some of them, such as mollies (*Poecilia latipinna*), guppies (*P. reticulata*), or swordtails and platies (*Xiphophorus spp*) are very popular for the aquarium trade, and are distributed around the world, becoming invaders upon escape from aquaria (Duggan et al., 2006). Others such as *Gambusia holbrooki* and its sibling *G. affinis* have been introduced worldwide as biological control agents for mosquito populations acting as malaria vectors (Krumholz, 1948; Pyke, 2005). Irrespective of the reasons for their introductions, invasive poeciliids have well-known impacts on native biota (Gamradt and Kats, 1996; Pyke et al., 2008; Stockwell and Henkanaththegedara, 2011). Several ecological factors contribute to the invasive success of poeciliid fish, including high thermal and salinity tolerance (Stockwell and Weeks, 1999), a short period for population recovery (Chapman and Warburton, 2006; Deacon et al., 2011), multiple paternity (Neff et al., 2008), and high dispersal capabilities (Rehage and Sih, 2004). Rapid evolution in life-history traits has been reported in newly founded populations of guppy *P. reticulata* (Reznick et al., 1990, 1997) and among recently translocated populations of mosquitofish (*G. affinis*) from shared ancestors in Hawaii (Stearns, 1983) and North-America (Stockwell and Vinyard, 2000). Such divergences among introduced populations sometimes reflect environmental adaptation (Reviewed in Reznick and Ghalambor, 2001) and can result in diverse impacts on ecosystem structure and functioning (Bassar et al., 2010).

5

Discussion

5.1 Genetic diversity in introduced populations

Lower genetic diversity is expected as a consequence of bottlenecks and reduced effective numbers following introductions (Frankham, 2005; Roman and Darling, 2007). Our results in Chapters 1, 2 and 3 add evidence of such a pattern for Spanish populations of mosquitofish when compared with those at the core of the natural distribution in North America. Previous studies on introduced populations of *G. holbrooki* agreed with these expectations both in Spanish (Vidal et al., 2010), Italian (Grapputo et al., 2006) or Australian locations (Ayres et al., 2010). Although, the origin of the Australian populations remains unclear (Ayres et al., 2013), Italian populations were likely founded in 1922 from introduced individuals in Spain (Sella, 1929). Historical records support the hypothesis of the sequential colonization of mosquitofish from America to Spain in 1921, and from there to more eastern countries in a model of stepping-stone introductions (Krumholz, 1948). This pattern should have led introduced mosquitofish populations to suffer broadly traceable losses of genetic diversity from native locations to the last areas of introduction (Grapputo et al., 2006). Detected genetic diversity losses in the introduced populations of the sibling species *G. affinis* agree with the recorded sequential pattern of introduction since the early 20th century from North-American native areas to the Hawaiian islands and then to the Philippines and New Zealand. In the New Zealand populations, a loss of 50% of allele richness and 25% on heterozygosity relative to native populations from Texas were attributed to this stepping-stone colonization process (Purcell et al., 2012). However, in *G. holbrooki*, reduced diversity and singular mtDNA haplotypes have been already reported in the northernmost American drainages originating during the postglacial colonization from southern refuge populations in Georgia or Florida (Scribner and Avise, 1994). A recent work based on microsatellite variation confirmed an important reduction in allele richness (up to 50%) and heterozygosity (up to 30%) of American *G. holbrooki* populations in North Carolina and northward compared to populations that occurred to the south in South Carolina and Florida (Sanz et al., 2013). Historical records suggest Edenton in North Carolina as the source location of the first European introduction of mosquitofish, and mtDNA sequence

comparisons corroborated this suggestion (Vidal et al., 2010). In Chapter 1, the levels of genetic diversity in the studied Spanish populations were compared with those observed in northern American mosquitofish populations considered as putative sources of the fish introduced to Europe. Surprisingly, the averaged diversity in the American populations from North Carolina (Brunswick) and Washington (Potomac) (H_S = 0.552) agreed fairly well with the estimated total diversity in the studied Spanish basins (H_T = 0.549). These results suggested limited or negligible losses of neutral genetic variation in the introduced populations of the Iberian Peninsula, as suggested for European populations by Sanz et al. (2013), and in agreement with the results reported from an American-European SNP diversity survey (Vidal et al., 2012). The discrepancies between our findings and those in Grapputo et al. (2006) could be then related to the collection from Florida used as American reference in this earlier work. Nevertheless, allele richness is reduced in some Spanish populations (Table 5.1) probably because this parameter is more sensitive than heterozygosis to bottlenecks and founder effects (Leberg, 1992; Spencer et al., 2000). Yet substantial levels of heterozygosis are still retained despite reiterate founder-flush events involving low effective numbers, such as the only 12 individuals having initially been introduced in Spain in 1921 (Chapter 1). Strong inter-annual fluctuations in effective population sizes were not an obstacle to maintaining genetic diversity levels among different generations (Chapter 3).

Table 5.1: Levels of diversity in *G. holbrooki* and *G. affinis* populations belonging to different native and invaded regional areas.

Area	A_R	H_E	Reference
Muga, Fluvià and Ter Rivers (Spain)	2.83	0.44 (0.35 - 0.5)	Chapter 1
Ebro River (Spain)	3.67	0.52 (0.49 - 0.56)	Chapter 2
Millars, Júquer and Segura Rivers (Spain)	3.64	0.57 (0.53-0.59)	Sanz et al. 2013
Tajo River (Portugal)	3.16	0.46	Sanz et al. 2013
Potomac and Brunswick (USA)	3.87	0.56 (0.54 - 0.58)	Chapter 1
Florida (USA)	10.57	0.87	Sanz et al. 2013
South Carolina (USA)	-	0.69 (0.67 - 0.7)	Zane et al. 1999
Great Melbourne area (Australia)	2.4	0.41 (0.21 - 0.53)	Ayres et al. 2010
Mississippi (USA) (*G. affinis*)	7.6	0.66	Sanz et al. 2013
Texas (USA) (*G. affinis*)	11.9	0.75	Purcell et al. 2012
North Island (New Zealand) (*G. affinis*)	4.4	0.61 (0.53 - 0.68)	Purcell et al. 2014

Because the additive genetic variance is less sensitive to bottlenecks than neutral variation (see reviews in Hänfling and Brandl, 2002; Blanchet, 2012), it is likely that the introduced Spanish mosquitofish populations substantially maintain the evolutionary potential already present in the American sources. Other invasive species undergoing similar processes, such as the introduced Australian populations of the guppy (*Poecilia reticulata*), showed strong genetic bottlenecks in genetic diversity when measured with neutral markers as microsatellite loci, yet these populations retained substantial additive variation (Lindholm et al., 2005). Reduced genetic diversity was observed among the Japanese populations of bluegill (*Lepomis maculatus*) consistent with their origin from a single imported American stock; nevertheless, these fish successfully spread throughout Japan (Kawamura et al., 2010). Similarly, Hawaiian populations of *G. affinis* retained or

even incremented their levels of allele richness and heterozygosity following their introduction (Scribner et al., 1992) and displayed significant divergence when compared with the ancestral Texas populations for a number of life-history traits (Stearns, 1983). Despite the bottleneck following introduction, enough additive variance to respond to novel selection pressures was probably conserved in the *G. holbrooki* population introduced to Spanish basins, favouring its successful and quick circum-Mediterranean expansion. Latitudinal (among basins) and longitudinal (within basin) variation has been observed in life-history traits such as reproductive effort and body condition among the Spanish *G. holbrooki* populations (Carmona-Catot et al., 2011). The Atlantic mosquitofish populations in France also diverged from the Mediterranean populations in thermal tolerance for growth and male length at sexual maturity (Beaudouin et al., 2008). Additionally, (Benejam et al., 2008) identified only a single species of parasite in the introduced Spanish populations in contrast with more than 50 known parasites in the native areas of America. Although a reduction in number of parasite is a general trend in invasive species due to the enemy release hypothesis, the reduction to a single parasite contrasts with evidence from other introduced areas such as Australia, where up to 11 species of parasites have been detected (Dove, 2000).

As suggested in Chapter 1, we consider that mosquitofish populations in the postglacially colonized areas in America may have acted as invasive bridgeheads that gained its invasive potential during the evolutionary events related with the northward expansion of mosquitofish after glacial retreat. Although identifying invasive bridgehead populations may be limited to wide-ranging and highly detailed studies of invaders, there is a growing number of recognized invaders following similar patterns, especially among agricultural pests (see Guillemaud et al., 2011). One of the most illustrative cases is the worldwide spread of the harlequin ladybird (*Harmonia axyridis*). This species is native from Southeast Asia, and there are clear signals that a well-established invasive American population was the bridgehead for the new invasive populations in South America, South Africa, and South Europe (Lombaert et al., 2010, 2011; Tayeh et al., 2013). Certainly, populations founded after the retreat of glacial sheets often display less genetic diversity as a consequence of the colonization process (see for example Bernatchez and Wilson, 1998). However, they can develop the ability to adapt to changing conditions by quickly recolonizing new areas in repeated founder-flush cycles, as shown by Bernatchez and Dodson (1991) for the lake fish *Coregonus clupeaformis*. Likely, these postglacial established populations conserve or promote adaptive variance despite overall losses of genetic diversity. In addition, despite the fact that populations situated at the limits of the native distribution range of the species usually display lower diversity levels than populations at the core of the distribution, they often show greater stress-adaptation favoring subsistence in unstable environments, and were source locations in a number of invasive species (revised in Lee and Gelembiuk, 2008). For instance, unstable environments and Pleistocene glaciations may have been involved in the appearance of the invasive populations of the zebra mussel, *Dreissena polymorpha*. The Ponto-Caspian populations of the species subjected to unstable environmental conditions during Pleistocene climatic fluctuations were the sources of invasive populations introduced elsewhere, meanwhile other *Dreissena* species inhabiting more ancient and stable lakes

have not been reported as being invaders (Gelembiuk et al., 2006). Altogether, these observations support our hypothesis indicating that despite displaying lower levels of genetic diversity, mosquitofish populations currently inhabiting the northernmost American basins acquired the evolutionary shifts necessary to become invasive during the post-glacial period, a long time before the European introductions. We therefore believe that this bridgehead hypothesis could be applied to other invasive species, specially those arising from unstable environments such as post-glacially colonized areas.

5.2   Patterns of differentiation among introduced populations

Classical studies using allozyme markers indicated large genetic divergence among American mosquitofish populations (F_{ST} = 0.175 - 0.270, Hernandez-Martich et al., 1995), being among the most observed within fish species with similar geographical distributions (Hernandez-Martich and Smith, 1990), such as *Lepomis macrochirus* (F_{ST} = 0.029, Avise and Felley, 1979), or from other territories (F_{ST} = 0.080 in *Gasterosteus aculeatus*, Baumgartner, 1986). There is not a global estimation of population differentiation for microsatellite loci among native populations of *G. holbrooki* other than those calculated in Chapter 1 (F_{ST} = 0.242) and in Sanz et al. (2013) (F_{ST} ∼ 0.25), both based in a few American collections. The close-related guppy (*Poecilia reticulata*) displayed a similar amount of differentiation at microsatellite loci (F_{ST} ∼ 0.3) among populations in the native areas of Trinidad and Tobago (Neff and Fraser, 2010). An SNP survey between American and European populations of mosquitofish also detected similar levels of differentiation (F_{ST} ∼ 0.2) either among American or among European populations of *G. holbrooki* (Vidal et al., 2012). Between all Spanish populations studied in this thesis pairwise population differentiation ranged between 0 and 0.337, with an average F_{ST} of 0.143. These values agree with those observed by Sanz et al. (2013) among a range of Mediterranean Spanish populations (average F_{ST} = 0.165, range 0.026 - 0.255). However, higher levels were detected among other introduced populations in Europe (average F_{ST} = 0.220, Sanz et al., 2013), and Australia (average F_{ST} = 0.269, Ayres et al., 2013), but both surveys involved more distant locations (over 1000 km) than the ones in our studies (300 km). In the Great Melbourne Area of Australia, where the farthest populations are ∼ 100 km apart, the differentiation levels obtained for introduced *G. holbrooki* were also higher (global F_{ST} = 0.239) than those among the Spanish locations in our study region (Ayres et al., 2010). Nevertheless, among the *G. affinis* populations introduced in the North Island of New Zealand with populations located up to 600 km apart, detected levels of differentiation (average F_{ST} = 0.180, range 0.017 - 0.330) were closer to the ones observed in our results (Purcell and Stockwell, 2014). Combining information from Chapters 1 and 3, the results clearly showed larger divergence between the American samples collected 500 km apart (PO and BW) than the studied Spanish locations (Figure 5.1). Thus, although presenting significant evidence of population structure, the levels of differentiation at the regional scale in the studied Spanish basins were lower than in other areas where the species is native and lower or similar to other introduced populations.

Population relationships depicted in Figure 5.1 also pointed to the lowland locations of the Ter (TV and CL) and Fluvià (PP) as the first ones where mosquitofish were introduced
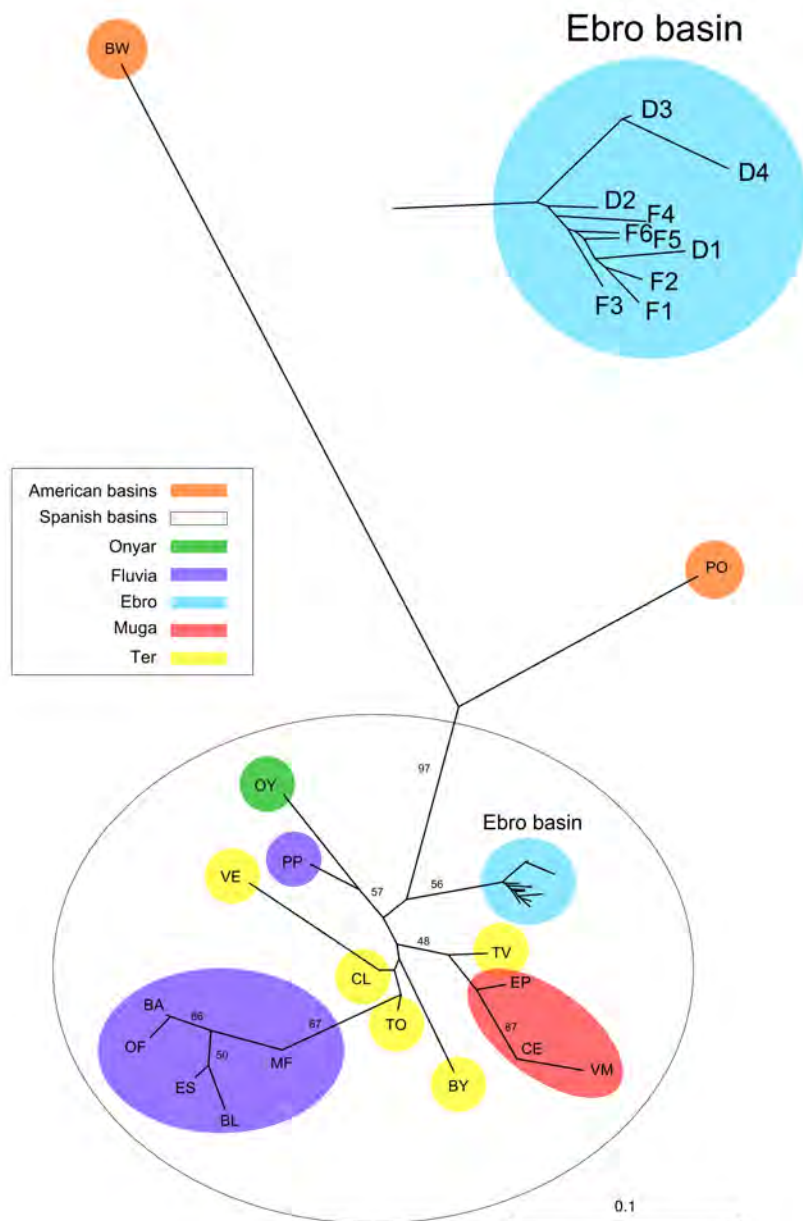
Figure 5.1: NJ tree of Nei's D$_A$ distance among all *G. holbrooki* Spanish populations studied in this work and including two American populations suggested as geographically close to the sources of the European introduction. Colours indicate different basins. Bootstrap values above 50 are shown. Location codes as in Chapters 1, 2 and 3.

in the northernmost Mediterranean basins of the Iberian Peninsula, likely from the fish of the Ebro basin. This figure also suggests higher divergence among the northernmost Spanish populations than among the Ebro collections even though some sampled locations in the Ebro River were separated by up to 23 km. The levels of genetic differentiation estimated in Chapter 2 for populations within the Ebro River (F_{ST} = 0.016) were the lowest among the basins we studied, and in the northernmost locations, F_{ST} values ranged from 0.042 at the Muga River to 0.116 at the Ter River. *G. holbrooki* from native areas showed patterns of population structuring usually manifesting higher divergences between than within rivers (see Hernandez-Martich and Smith, 1990). Analyses on the closely related species *Heterandria formosa* in Florida, suggested little spatial subdivision along rivers (F_{SR} = 0.015) but higher among them (F_{RT} = 0.074, Baer, 1998); similar results were described among regional populations of the green swordtails, *Xiphophorus helleri*, in Belize (Tatarenkov et al., 2010). AMOVA analysis including all studied Spanish populations assigned higher differentiation among (13.1%) than within (4.1%) river basins (Table 5.2). Larger population divergences among rivers than within them have also been detected in other species in the same geographical range. For example, analyzing Mediterranean populations of threespine stickleback (*Gasterosteus aculeatus*) in a region similar to the ones in our study, Araguas et al. (2012) estimated that the proportion of genetic variance explained by differentiation among basins was three fold the diversity among locations (32.95% vs. 10.8%). A similar pattern has been estimated for the Iberian Mediterranean endemic killifish (*Aphanius iberus*) populations (Araguas et al., 2007). Nevertheless, results of Table 5.2 likely reflect the divergence between the Ebro mosquitofish and collections from the other river basins. AMOVA in Chapter 1 assigned slightly lower differentiation within (8.8%) than among (10.3%) northern basins, but in Chapter 3, the opposite results were obtained. Discrepancies between these two chapters maybe the result of the distinct set of locations used in both studies, and the results roughly suggest similar levels of differentiation within than among studied basins (8-10%). As indicated in Chapter 3, annual flooding periods represent greater opportunities for gene flow between basins in the lowland areas, but also contribute to divergence between upstream and lowland populations within basins. Despite evidencing high levels of dispersal, the fine-scale population pattern in the region was stable over time (Chapter 3). Detected low levels of differentiation within rivers in Spanish mosquitofish populations are consistent with observed patterns in the native range where large transects along river basins were occupied by highly related populations (McClenaghan et al., 1985; Smith et al., 1989).

Table 5.2: Hierarchical analysis of molecular variance (AMOVA) of microsatellite variation among all *G. holbrooki* Spanish populations studied including basins from the Empordà area and the Ebro River. $***$: *P-value* < 0.001

	Fixation indices	Percentage of variation
Among basins	0.131 $F_{CT}***$	13.1%
Among populations within basins	0.048 $F_{SC}***$	4.1%
Within populations	0.173 $F_{ST}***$	82.7%

5.3   Evolutionary forces driving fine-scale population structure

Hernandez-Martich et al. (1995) attributed the differentiation among *G. holbrooki* populations in America to: (1) the effect of selective processes acting at microgeographical ranges favouring certain specific genetic adaptation to local conditions at both regional and local scales (already indicated in McClenaghan et al. (1985)) and (2) differential genotype dispersal patterns. Genetic drift was discarded in that case due to the absence of severe bottlenecks and founder events for the populations in the studied area. Selection processes have been demonstrated to promote variation in mosquitofish populations inhabiting coastal marshlands as result of periods of saltwater inundation (Congdon, 1994, 1995) or from exposure to pollutants such as arsenate (Newman et al., 1989). Adaptive differentiation can explain geographical variation in the mosquitofish populations of the Mediterranean area for life-history traits such as reproductive effort, body condition (Carmona-Catot et al., 2011), and thermal tolerance (Beaudouin et al., 2008). In the mosquitofish populations of the Ebro River, we detected allelic variation at the GPI-2 locus in response to acute mercury concentration with relevant effects on the local population structure in the Flix Reservoir (Chapter 2). Microsatellites confirmed the most mercury exposed location in the reservoir as a sink for fish originated in neighbouring locations.

Contrasting with observations in American mosquitofish populations, the analyses carried out in Chapter 1 pointed to bottleneck signals within many Spanish populations associated with the introduction and colonization histories. A similar situation had probably occurred in the introduced Australian *G. holbrooki* populations, in which founder events and repeated bottlenecks related to introductions, drought impacts, and overwintering effects likely explained an important part of the observed genetic population structure (Ayres et al., 2013). Nevertheless, inter-annual effective population size fluctuations did not result in substantial alterations on the gene diversity pattern in the study's northernmost mosquitofish populations (Chapter 3). As observed along American rivers, unidirectional downstream gene flow would cause more differentiation, lower heterozygosity, and fewer alleles in upstream than in downstream G. holbrooki populations (Hernandez-Martich and Smith, 1997). We detected increased diversity and lower differentiation in lowland populations (see Chapters 1 and 3) suggesting that downstream dispersal and increased connectivity potential through irrigation diversions (Chapter 3) were probably a general tendency influencing the mosquitofish fine-scale population structure.

Distance-limited dispersal can lead to geographical clustering and an isolation-by-distance population structure (Carlsson et al., 2004). In America, populations where *G. holbrooki* is native evidence of this pattern of differentiation has been attributed to short-distance dispersal along rivers (Hernandez-Martich and Smith, 1997). In an analysis of the *G. holbrooki* temporal variation of Florida populations, McElroy et al. (2011) suggested that regional IBD patterns were related to a dynamic spatial structure in response to hydrological events (floods and droughts). Among the *G. hubbsi* inhabiting the blueholes of Andros Island (Bahamas), however, the lack of IBD was related to the differentiation pattern of an island model with small populations receiving gene flow from a big panmictic source population (Schug et al., 1998). IBD has also been detected at

regional scales in the introduced Spanish (Vidal et al., 2012) and Australian populations of mosquitofish (Congdon, 1995; Ayres et al., 2010). In the study region, the divergence among populations at small regional scales agrees with a two-dimensional IBD pattern, as described in Chapter 1. Such a pattern reflects both the natural movement of individuals within and among rivers and the human-mediated transport of individuals between rivers favoured by an complicated two dimensional network of roads. Ayres et al. (2010) obtained a rather similar pattern in introduced areas of Australia, where gene flow among river basins was the result of human-mediated transport. However, among mosquitofish populations, IBD is often weak within river basins (Chapter 1; Ayres et al., 2010; Purcell and Stockwell, 2014). Within rivers, IBD patterns depend on the connectivity among populations (Ayres et al., 2010) and other factors limiting gene flow such as the presence of physical barriers between them (Crispo et al., 2006) or biological agents as predators (Shaw et al., 1994). Studies in basins from native mosquitofish distribution range showed a one-dimensional pattern of IBD caused by directional dispersal along rivers (Hernandez-Martich and Smith, 1990, 1997). Prevalent downstream gene flow has also been observed in populations of *G. holbrooki* in introduced populations of Australia (Congdon, 1995), but also detected a preference of dispersal between neighbouring locations along the river produced a pattern of one-dimensional IBD, as observed among the Ebro locations (Chapter 2).

A large proportion of immigrant fish within each Spanish location suggested by the Bayesian estimates of contemporary gene flow and assignation tests in Chapters 1, 2 and 3 also contribute to maintain levels of diversity within locations. *G. holbrooki* can disperse up to several kilometers a year (Alemadi and Jenkins, 2007). Contrary to the pattern of reduced short-distance dispersal detected for invasive populations of *G. affinis* in New Zealand (Purcell and Stockwell, 2014), our results showed a large proportion of local individuals originated from populations located in the same or even other river basins. However, we have been unable to directly identify gene flow rates, indicating a preference of downstream over upstream movement in our studies in Spanish populations. In fact, in Chapter 2 we detected evidence of active upstream dispersal among close populations within the Ebro River, even to surpass the Flix dam. Also in the Ebro River, the two farthest populations allocated downstream displayed significant rates of upstream dispersal between them. As observed along rivers of American *G. holbrooki* populations, unidirectional downstream gene flow causes more differentiation, lower heterozygosity, and fewer alleles in upstream than in downstream populations (Hernandez-Martich and Smith, 1997). In the Spanish basins studied we detected increased diversity and lower differentiation in lowland populations (Chapter 1), suggesting that downstream dispersal and increased connectivity through irrigation channels (Chapter 3) are, in fact, a general tendency along these invaded rivers.

6

Conclusions

1. Local levels of genetic diversity in invasive populations of *G. holbrooki* in NE Spanish Mediterranean streams are generally lower than populations at the core of the native area of distribution in North America, but agree with those described in locations close to sources of the European introduction.

2. Introduced European *G. holbrooki* populations likely retained the evolutionary potential that was acquired for the North American sources probably during post-glacial colonization.

3. Genetic diversity levels were similar among locations from the different river basins studied, and inter-annual fluctuations on effective population size were not an obstacle for these populations to retain levels of diversity over time.

4. In the Spanish NE Mediterranean rivers, hydrography and connectivity were the main drivers of differentiation, producing significant structuring among populations. Despite reiterate droughts and floodings, fine-scale population structure was stable among generations.

5. Regional mosquitofish populations showed larger population divergences among than within rivers. At lower scales, local processes obscured such patterns by increasing population divergences within basins

6. Large proportions of immigrants contributed to local populations every year, reflecting the high dispersal potential of the species. These migrating fish contributed to genetic exchanges between locations and to maintaining levels of diversity in the entire basin.

7. In some river basins, a one-dimensional IBD differentiation pattern arises as consequence of the natural linear fish dispersal. High dispersal potential also contributes to the spread and colonization of suitable habitats throughout the entire river basin following the initial introduction.

8. As observed in the Ebro River, neither pollution nor dams prevent mosquitofish dispersal along river. Even more, water diversions in the lowland plain of the northernmost rivers, promotes fish exchanges between basins.

9. The two-dimensional IBD pattern of population divergence observed at regional scales likely resulted from human disturbances of habitat (i.e., increasing connectivity) and also from fish translocations between basins. Human-assisted translocations promote opportunities for colonization of new environments like the upstream reaches of the rivers.

7

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