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Origin and genetic diversity of mosquitofish (*Gambusia holbrooki*) introduced to Europe

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Abstract We provide mitochondrial sequence variation of the invasive fish *Gambusia holbrooki* from 24 European populations, from Portugal to Greece. Phylogeographic structure in Europe was compared with genetic data from native samples (USA) and historical records were reviewed to identify introduction routes. Overall, data agree with records of historical introductions and translocations, and indicate that the most abundant haplotype throughout Europe originated from North Carolina and corresponded to the first introduction in 1921 to Spain, being transferred to Italy in 1922 and to many countries afterwards. Our results also show that at least another independent introduction occurred first in France and subsequently from France to Greece. Haplotypes of *G. affinis* were not detected in our European sampling effort but historical records and other data suggest that this species was introduced to Italy in 1927 and it might be present. At the continental scale, there is less diversity in Europe than in North America, in agreement with the low number of introduced fish. At the local scale, some European populations gained diversity from multiple introductions and from "de novo" mutations.

Keywords Invasive species · *Gambusia holbrooki* · *Gambusia affinis* · Genetic diversity · Mosquitofish

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Introduction

Beyond their negative effects, invasive species are an underappreciated opportunity to study ecology and evolution at unusually large spatial and temporal scales (Rice and Sax 2005). For instance, two paradoxes of biological invasions that should help to improve ecological and evolutionary theories are: (1) why do invasive exotic species colonize and displace native species that should be better adapted to local environments? (Sax and Brown 2000; Allendorf and Lundquist 2003); and (2) why do invasive species flourish despite reduced genetic diversity in the recipient region? (Allendorf and Lundquist 2003). To answer these questions we need to increase our knowledge on invasive species and assess its genetic structure and variability within the invaded environment.

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Two closely related poeciliid species, *Gambusia holbrooki* Girard, 1859 and *Gambusia affinis* (Baird and Girard, 1853), are only native to the United States and Mexico but have been introduced into more than 50 countries (García-Berthou et al. 2005) in order to control mosquito populations and hence malaria (Krumholz 1948). Collectively, these two species are considered among the most invasive fish, with well known effects in the decline and local extinction of native amphibians and fishes (Courtenay and Meffe 1989; Kats and Ferrer 2003; Alcaraz et al. 2008). However, the worldwide distribution of these two species is still largely unclear (Pyke 2008), partly because of taxonomic confusion. In the early twentieth century, when mosquitofish were introduced worldwide, they were regarded as three separate species (with *G. patruelis*, which is now considered a synonym of *G. affinis*), later as two subspecies of a single species (*G. affinis*), and not until Wooten et al. (1988) as two separate species. Therefore, many records that refer to *G. affinis* are actually *G. holbrooki* (Haynes and Cashner 1995).

Apparently, both species were introduced to Europe in the 1920s (Krumholz 1948) and although mostly *G. holbrooki* is cited, it is unclear whether both species are present. According to historical records, *G. holbrooki* was first introduced to the Iberian Peninsula in 1921, was then transferred to Italy in 1922, being nowadays highly abundant in most Mediterranean countries (see e.g. Krumholz 1948). *G. affinis* arrived in Italy directly from the USA around December 1927 (Sella 1926; Anonymous 1927) but despite being cited, it is unclear whether it is still present in Europe, given the above-mentioned taxonomic problems.

Although the genetic diversity of both mosquitofish species has been thoroughly analyzed in the native populations of North America (Stearns 1983; Wooten and Lydeard 1990; Scribner and Avise 1994; Mulvey et al. 1995) and in *G. holbrooki* introduced to Australia (Congdon 1994, 1995), the only study in Europe is Grapputo et al. (2006), performed only on four collections from Italy and Spain. Interestingly, founder events and population bottlenecks in early stages of introductions, which are considered responsible for the loss of diversity of many invasive species (Allendorf and Lundquist 2003; Roman and Darling 2007; Dlugosch and Parker 2008; Suarez and Tsutsui 2008), were observed in these four European mosquitofish populations.

The main objectives of this paper are: (1) to assess the genetic structure of mosquitofish throughout Europe and check for the presence of *G. affinis* in this continent, and (2) to compare genetic diversity and population structure of *G. holbrooki* to those found in its native range. *G. affinis* and *G. holbrooki* show substantial geographic genetic structure in their native range (Wooten et al. 1988; Scribner and Avise 1993), so by comparing the genetic diversity of European and North American populations and incorporating information from the historical records of introductions we expect to confirm multiple geographical origins and the routes of introduction.

Materials and methods

Collections

A total of 417 individuals of *G. affinis* or *G. holbrooki* from 33 locations were screened for sequence variation at the cytochrome *b* gene of the mitochondrial genome. Nine locations were in North America (the native region of mosquitofish), and included 30 putative specimens of *G. affinis* from the Big Black River (Mississippi drainage) kindly provided by the Mississippi Museum of Natural Science. The remaining 24 locations corresponded to introduced European populations in Spain (9 samples), Portugal (4), France (5), Italy (2), Hungary (1), and Greece (3) (Table 1; Fig. 1). Further data on most of the Spanish and French samples are given elsewhere (Benejam et al. 2009).

DNA extraction, amplification and sequencing

Total DNA extraction was performed with *Chelex*® *100 Resin* (Biorad), similarly to the method described by Estoup et al. (1996). Approximately 30 mg of muscular tissue from each individual were digested with 200 µg of proteinase K in 500 µl of *Chelex* 10% at 65°C for 1 h and subsequently centrifuged at 13,000 rpm during 15 min. Primers CytBF1 (5′-ATG GCC AAC CTA CGA AAA AC-3′) and CytBR1 (5′-GGG TAG RAC ATA ACC TAC GAA G-3′) were designed in conserved regions of cytochrome *b* (*cytb*) gene based on GenBank sequences of *Gambusia*

Table 1 Location of the studied populations (with latitude and longitude; all latitudes are north), location code (*L*) and haplotype composition

Location	*L*	Country	Latitude	Longitude	Haplotype composition	h[a]	d[a]
Big Black River, Mississippi	1	USA	33°23′	89°37′ W	7 Hol1, 22 Aff1, 1 Aff4	0.4207 (0.0874)	0.0182 (0.0100)
Everglades, Florida	2	USA	25°26′	80°46′ W	18 Hol7, 2 Hol8	0.1895 (0.1081)	0.0012 (0.0014)
Gainesville, Florida	3	USA	29°39′	82°21′ W	20 Hol7		
Florence, South Carolina	4	USA	36°1′	79°57′ W	20 Hol4		
Brunswick, North Carolina	5	USA	34°17′	78°29′ W	15 Hol1, 1 Hol2	0.1250 (0.1064)	0.0004 (0.0007)
Pomona, New Jersey	6	USA	39°28′	74°34′ W	20 Aff1		
Lanoka harbor, New Jersey	7	USA	39°51′	74°11′ W	20 Aff1		
San Saba River Texas	8	USA	30°55′	99°47′ W	8 Aff2, 2 Aff3	0.3556 (0.1591)	0.0023 (0.0021)
Potomac River, Washington D.C.	9	USA	38°39′	77°11′ W	19 Hol1, 2 Hol6	0.1810 (0.1044)	0.0006 (0.0009)
Figueira da Foz, Mondego basin	10	Portugal	40°5′	8°45′ W	10 Hol1		
Ribeira de Alcáçovas, Sado basin	11	Portugal	38°23′	8°9′ W	10 Hol1		
Tapada, Tagus basin	12	Portugal	38°26′	9°7′ W	10 Hol1		
Ribeira da Lena, Lis basin	13	Portugal	39°42′	8°50′ W	9 Hol1, 1 Hol6	0.2000 (0.1541)	0.0006 (0.0009)
River Millars	14	Spain	39°56′	0°03′ W	10 Hol1, 2[b] Hol3	0.3030 (0.1475)	0.0010 (0.0012)
Altea	15	Spain	38°36′	0°02′ W	10 Hol1		
Ebro delta, Ebro basin	16	Spain	40°42′	0°49′ E	10 Hol1		
Lake Banyoles, Ter basin	17	Spain	42°7′	2°45′ E	10 Hol1		
River Fluvià	18	Spain	42°10′	3°04′ E	10 Hol1		
River Ter	19	Spain	42°01′	3°09′ E	10 Hol1		
River Segura	20	Spain	38°06′	0°39′ W	10 Hol1		
River Júcar/Xúquer	21	Spain	39°10′	0°17′ W	10 Hol1		
Minorca	22	Spain	40°02′	3°55′ E	10 Hol1		
Lacroix Falgarde, Garonne basin	23	France	43°31′	1°25′ E	10 Hol5		
Vistre, Rhône basin	24	France	43°36′	4°13′ E	8 Hol1, 2 Hol5	0.3556 (0.1591)	0.0012 (0.0014)
River Bourdigou	25	France	42°44′	2°59′ E	10 Hol1		
River Orb	26	France	43°15′	3°18′ E	10 Hol1		
Brière, Loire estuary	27	France	47°22′	2°19′ W	10 Hol1		
Coltano	28	Italy	43°38′	10°24′ E	10 Hol1		
Catania, Sicily	29	Italy	37°24′	15°3′ E	10 Hol1		
Lake Héviz	30	Hungary	46°37′	17°10′ E	10 Hol1		
Lake Pamvotis	31	Greece	39°41′	20°52′ E	5 Hol1, 5 Hol5	0.5556 (0.0745)	0.0018 (0.0018)
Anthili	32	Greece	38°50′	22°27′ E	2 Hol1, 8 Hol5	0.3556 (0.1591)	0.0012 (0.0014)
Rhodes	33	Greece	36°10′	27°59′ E	10 Hol1		

For each locality, the number of fish for the different *cytochrome b* haplotypes found in this study (see Table 2) is detailed. Haplotype (h) and nucleotide (d) diversities are also shown (SE within parentheses)

[a] Only values distinct from 0 are indicated

[b] Both individuals were heteroplasmic, showing the Hol1 and the Hol3 haplotypes

genus, including both *G. affinis* and *G. holbrooki* (see below). Amplification reactions had a final volume of 30 µl and contained 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer, 25 ng of genomic DNA and 0.3 units of Taq DNA polymerase (Ecogen S.R.L). The thermal profile included a first denaturing step at 94°C for 5 min followed by 35 cycles at 94°C (30 s), 50°C (2 min) and 72°C (2 min). PCR products were purified with the *ExoSAP-IT®* reagent (USB) and then sequenced with the *BigDye® Terminator v1.1 Cycle Sequencing kit* (Applied Biosystems) with PCR primer CytBF1. Clean sequences were obtained for a

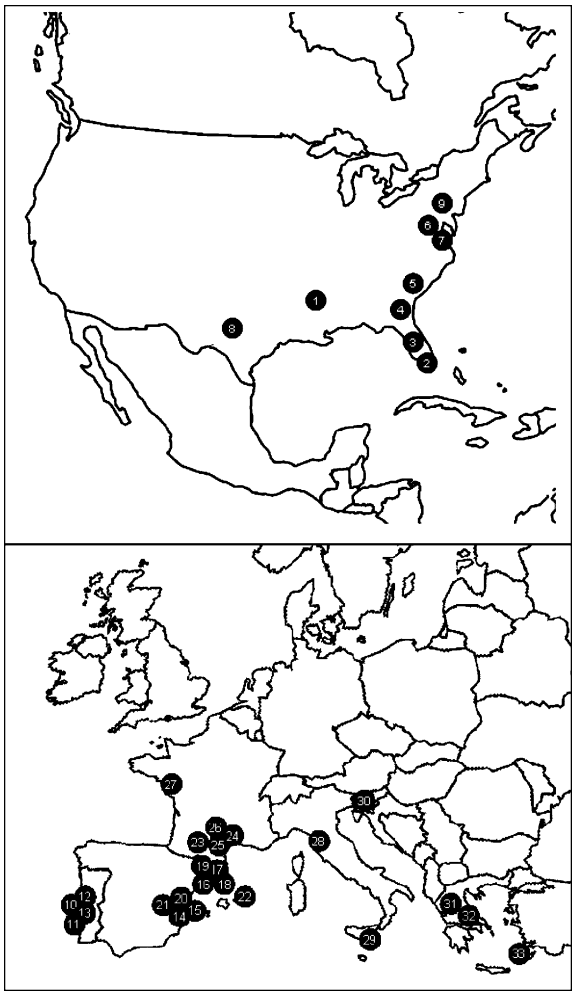

Fig. 1 Geographic location of *Gambusia* collections. See Table 1 for details on the location codes

fragment of 309 bp from site 86 (second position in codon 28) to site 394 (first position in codon 132) of the *cytb* gene.

Sequence and population analyses

To identify mosquitofish species introduced in Europe, European haplotypes were compared to the *Gambusia* cytochrome *b* sequences already available in GenBank. The GenBank data set included 21 sequences of *Gambusia* spp. and one outgroup species, *Belonesox belizanus*, described in Lydeard et al. (1994) (GenBank codes U18115.1, U18206.1 to U18209.1 and U18211.1 to U12228.1), three sequences of *G. affinis* and 3 of *G. heterochir* described by Davis et al. (2006) (GenBank codes DQ075681.1 to DQ075686.1) and four sequences of four other *Gambusia* species reported by Hrbek et al. (2007) (GenBank codes EF017514.1 to EF017516.1 and EF017518.1). All sequences were aligned using ClustalW Multiple Alignment accessory application implemented in the Bioedit software (Hall 1999) and the *G. affinis* EF017514.1 sequence as reference. For all sequences, further analyses were restricted to the aligned fragment of 309 bp corresponding to the amplified region of this study. *G. affinis* U18107.1 and *G. melapleura* U18216.1 sequences from Lydeard et al. (1994) were, however, shorter and only matched 268 and 307 bp, respectively, of our aligned fragment. Genetic distances were calculated using the Tamura–Nei method (Tamura and Nei 1993) with the number of base substitutions per site as units and the pairwise deletion option. The Tamura–Nei distance matrix was used to generate a neighbor-joining (NJ) tree (Saitou and Nei 1987) to infer the evolutionary relationships among sequences from this study and the GenBank ones (see above). Confidence values were estimated by 1,000 bootstrap replicates (Felsenstein 1985). This evolutionary sequence analysis was performed with the MEGA4 software (Tamura et al. 2007). In addition, a median-joining network (Bandelt et al. 1999) involving the *G. affinis* and *G. holbrooki* haplotypes was constructed using NETWORK 4.5.1.0 software (http://www.fluxus-engineering.com/sharenet.htm).

Genetic variation within collections was estimated by haplotype and nucleotide diversities (Nei 1987). Overall diversity present in North America and in Europe was estimated by pooling the data from each region. Patterns of haplotype and nucleotide diversity distribution among American and among introduced European collections were estimated by hierarchical analyses of molecular variance (AMOVA) of the frequency distribution of haplotypes (F_{ST}) and their Tamura–Nei pairwise divergence (N_{ST}) at two hierarchical levels: within and among collections within territories (USA or Europe). An additional AMOVA involving native American collections of *G. holbrooki* was performed using haplotype information from Scribner and Avise (1993). Although these authors did not provide haplotype frequencies, we assumed haplotype frequencies in their polymorphic collections to maximize intrapopulation diversity and to minimize population differentiation. The real F_{ST} values should be then greater than our computed

value, which represents an underestimate of the real divergence. However, because only 6 out of 29 collections were polymorphic in that study and samples sizes were small (a maximum of 4 fish per collection) our approach should not produce a strong bias. For instance, in a polymorphic population for two haplotypes we considered a frequency of 2:2 (for a sample of 4 fish) that, even if not being true, it is not far from the alternatives 3:1 or 1:3. All AMOVA computations were performed using Arlequin 3.11 software (Excoffier et al. 2005).

Results

Phylogenetic relationships among haplotypes

Sequence analyses resolved 12 haplotypes among the 417 analyzed fish (Table 2). Four of them were assigned to *G. affinis* because they clustered together with GenBank EF0175141 (Hrbek et al. 2007) and DQ075681.1 (Davis et al. 2006) *G. affinis* sequences. The other eight haplotypes grouped, with strong bootstrap support, with *G. holbrooki* GenBank sequence U18210.1 of Lydeard et al. (1994) (Fig. 2). Average number of nucleotide differences was 1.167 (±0.571) among *G. affinis* sequences and 2.000 (±0.774) among *G. holbrooki*. In the NJ tree, *G. affinis* and *G. holbrooki* haplotypes form monophyletic sister groups suggesting genetic distinctiveness of the two species. The average number of nucleotide differences observed between *G. affinis* and *G. hoolbroki* was 15.210 ± 3.431, while the average between all *Gambusia* species was 28.116 ± 2.940. The largest values were observed in pairwise comparisons involving any *Gambusia* species and the outgroup *B. belizanus* (50.872 ± 5.772).

Haplotype distributions and population diversity

Twenty-three out of 33 samples presented a single haplotype (Table 1). Haplotypes of *G. affinis* and *G. holbrooki* only co-occurred in the sample from the Big Black River, although *G. affinis* predominated. Haplotypes of *G. affinis* were only detected among American populations, whereas all examined European samples presented haplotypes of *G. holbrooki* (Fig. 3). The haplotype Hol1 was the only one found in sampled individuals from 18 European collections. The Hol5 haplotype was abundant in the two continental Greek samples and in two French collections. In Lacroix Falgarde from the Garonne River basin (France), all analyzed fish had this haplotype. Haplotype Hol5 distinguished from Hol1 by a GA transition in the first position of codon 123, generating the aminoacid change of Valine (Hol1) to Isoleucine (Hol5), with both aminoacids being nonpolar. The Hol3 haplotype was only detected in two heteroplasmic individuals collected in the Millars location, also carrying the Hol1 haplotype. These individuals were sequenced twice to corroborate their heteroplasmy. The nucleotide change that distinguished Hol1 and Hol3 haplotypes was a TC transition in a first codon position generating a change from the nonpolar Proline aminoacid (Hol1) to the polar Serine (Hol3). All the other nucleotide changes among *G. holbrooki* haplotypes were transitions in the third codon position, not generating any aminoacid change according to the standard genetic code.

Among introduced European populations, haplotype diversity was high in the two continental Greek collections (Pamvotis and Anthili) and one French population (Table 1). In America, the highest diversities were observed in the *G. affinis* population from the San Saba River and in the collection from the Big Black River in the Mississippi basin. This later collection also had the highest nucleotide diversity because of the co-occurrence of haplotypes of both *Gambusia* species. This result was unexpected since previous genetic analyses from the Mississippi River basin showed pure *G. affinis* populations in this basin (Scribner and Avise 1993). Our finding could be related to an ongoing recent contact between both species and perhaps hybridization.

Population structure

The Hol1 haplotype was abundant in sampled individuals from North Carolina and Potomac River, while the related Hol4 haplotype (Fig. 3) was the only one detected in fish sampled from South Carolina. The most different Hol7 haplotype (Fig. 3) was abundant in fish from Florida (Table 1). Consequently, a large level of population structure was observed among American collections, with 94% of diversity contributing to the differentiation among collections (ST values, Table 3). This amount of

Table 2 Variable positions in the 309 bp fragment of *cytochrome b* gene

Species	Sequence	Site number in the 309 bp fragment																						
		5	17	41	42	53	92	95	105	113	116	134	143	173	188	197	218	236	263	278	281	282	284	293
G. affinis	U18107.1	A	T	T	G	G	C	T	T	C	C	T	C	G	T	C	A	C	T	T	A	G	C	C
G. affinis	DQ075683.1				A					T														
G. affinis	EF017514.1				A																			
	DQ075686.1																							
G. affinis	DQ075681.1				A																G			
G. holbrooki	U18210.1	G	A		A	A	T	C				C	G	A	C		G	T		C			T	T
G. affinis	Aff1				A																			
G. affinis	Aff2				A																G			
G.affinis	Aff3				A					T														
G.affinis	Aff4				A														G					
G. holbrooki	Hol1	G	A		A	A	T	C				C	G	A	C	T	G	T		C			T	T
G. holbrooki	Hol2	G	A		A	A	T	C				C	G	A	C	T		T		C			T	T
G. holbrooki	Hol3	G	A		A	A	T	C	C			C	G	A	C	T	G	T		C			T	T
G. holbrooki	Hol4	G	A		A	A	T	C			T	C	G	A	C	T	G	T		C			T	T
G. holbrooki	Hol5	G	A		A	A	T	C				C	G	A	C	T	G	T		C		A	T	T
G. holbrooki	Hol6	G	A	C	A	A	T	C				C	G	A	C	T	G	T		C			T	T
G. holbrooki	Hol7	G	A		A	A	T	C				C	A	A	C			T		C			T	T
G. holbrooki	Hol8	G	A		A	A	T	C				C	G	A	C		G	T		C			T	T

GenBank sequences and haplotypes found in this study are presented

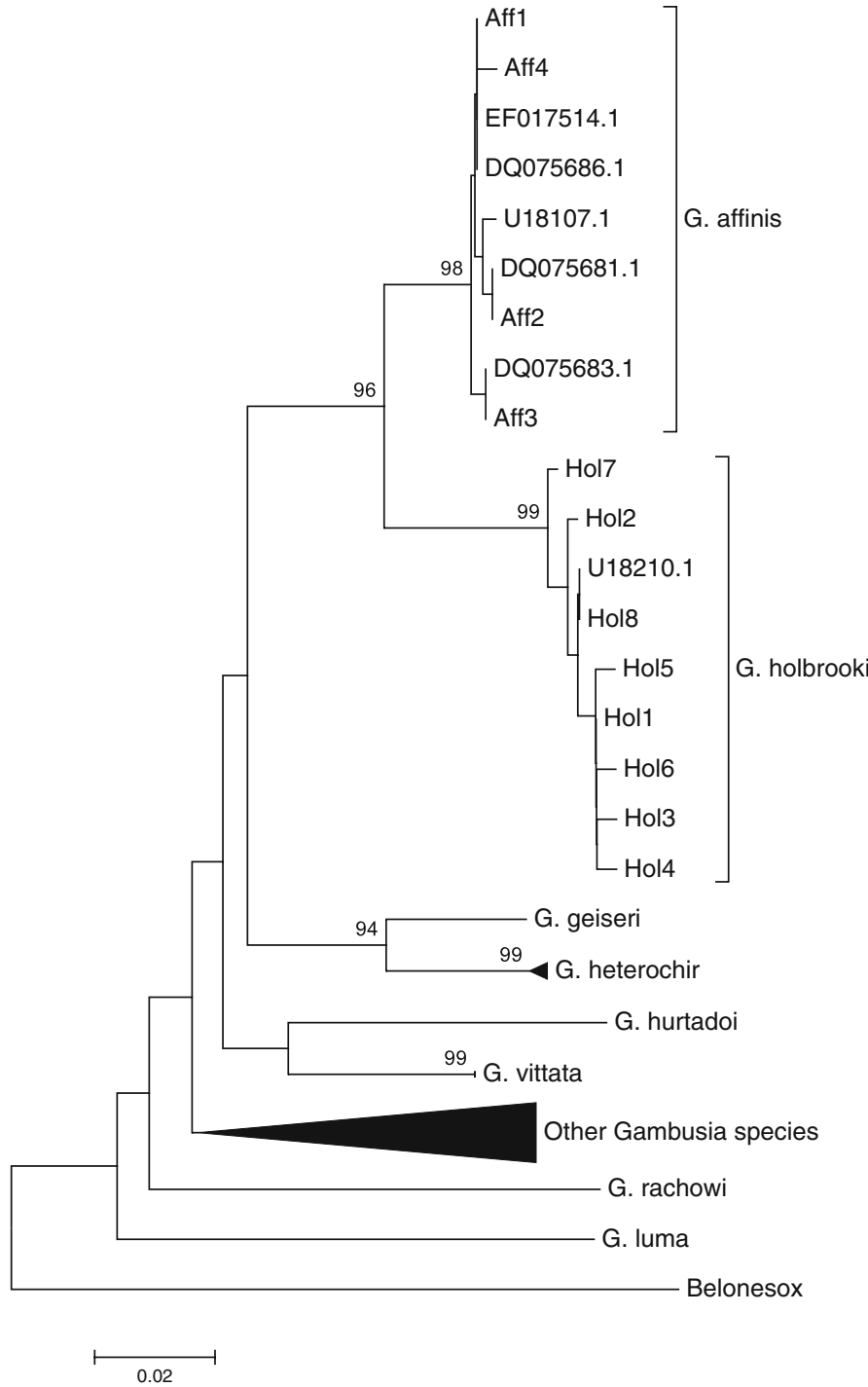

Fig. 2 Neighbor-joining tree showing the evolutionary relationships of 40 *Gambusia* taxa. Bootstrap values (over 1,000 replicates) higher than 90% are shown

population differentiation was greater than the observed in the review of Scribner and Avise (1993) probably because our sampling in America was limited to a single collection from contrasting river basins.

The Hol1 was the most abundant haplotype throughout Europe (Table 1, Fig. 3) and 18 out of 24 collections showed only this haplotype. Haplotype diversity was significantly higher in America than in Europe (Welch's t-test: $t_{318.1} = 14.3$, $P < 0.0005$)

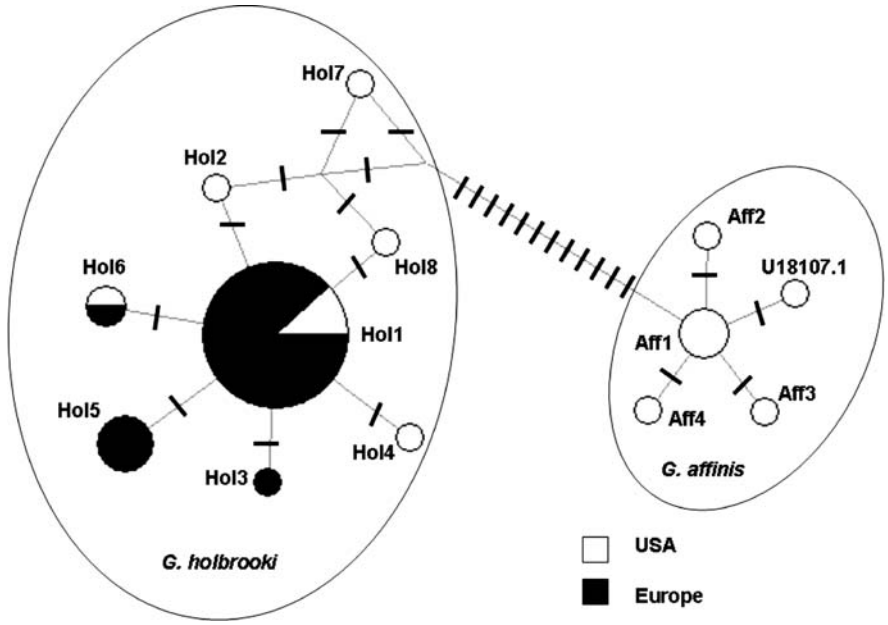

Fig. 3 Median-joining network involving all *Gambusia affinis* and *G. holbrooki* haplotypes. For haplotypes detected in this study the area of each circle is proportional to the number of European collections exhibiting that haplotype. Each *bar* in the network represented one mutational step

Table 3 Haplotype (*H*) and nucleotide (*N*) diversities (average and, between parentheses, standard error) and population differentiation (*ST*) in American and introduced European populations of *G. holbrooki*

Territory	Diversity parameter	Total diversity (SE)	Locations	*ST*	Source
USA	*H*	0.6873 (0.0210)	5	0.9382	This study
	H	0.7917 (0.0269)	29	0.8442	Scribner and Avise (1993)
	N	0.0059 (0.0040)	5	0.9382	This study
Europe	*H*	0.2081 (0.0260)	24	0.6490	This study
	N	0.0007 (0.0009)	24	0.6490	This study

but nucleotide diversity was not significantly different ($t_{105.9} = 1.27$, $P = 0.21$). In addition, divergence among European collections only accounted for 36% of total diversity and mostly reflected the presence of Hol5 in fish from some French and Greek locations (Table 3).

Discussion

Origin of mosquitofish introduced to Europe

Although our analyses only detected *G. holbrooki* throughout Europe, historical records indicate that both mosquitofish species were introduced. In 1921, *G. holbrooki* was first introduced to Cáceres (Spain) and from there to Italy and elsewhere, whereas *G. affinis* was originally introduced in Trieste (Italy) in 1927 (Krumholz 1948). Sella (1929), the main promoter of the first European introductions, explained that after the introduction in 1921 of *G. holbrooki* to Spain, *G. affinis* (referred to as *G. patruelis*) were introduced in December 1927 to ponds in Rovigno and Valle d'Istria in Italy from collections obtained from Carbondale (Illinois), because they were supposed to resist cold weather better than *G. holbrooki* (Sella 1926; Anonymous 1927). Moreover, a few recent papers cite simultaneously

G. affinis and *G. holbrooki* in Turkey (Ekmekçi and Kirankaya 2006; Innal and Erk'akan 2006), thus apparently discarding synonymy problems. Veenvliet (2007) identified mostly *G. holbrooki* but also a single male of *G. affinis* among Slovenian mosquitofish populations, although more recent data suggests that only *G. holbrooki* is present (P. Veenvliet, pers. comm.). In North America both species hybridize and *G. holbrooki* genotypes tend to outcompete and even replace *G. affinis* ones, where they coexist naturally or by introductions (Scribner 1993; Scribner and Avise 1994; Walters and Freeman 2000). In our analysis, we have not detected *G. affinis* and only *G. holbrooki* seems to be present throughout Europe. However, considering introduction reports and hybridization dynamics in the USA, the existence of *G. affinis* or introgressed individuals seems possible. We urge, nevertheless, to refer to the species in Europe as *G. holbrooki* in future literature, unless gonopodium morphology (Rauchenberger 1989), fin ray counts (Walters and Freeman 2000) or genetic identification clearly demonstrates that it is *G. affinis*.

Our results also suggest several independent introductions of *G. holbrooki* from at least two American sources, partially agreeing with historical records. All but one European populations surveyed in this study had the haplotype Hol1, abundant in native populations of North Carolina and northward. Although Krumholz (1948) and subsequent literature (without citing earlier references) reported that the 1921 introduction in Spain originated from Augusta (Georgia), Artom (1924) and Nájera Angulo (1944) stated that they came from Edenton (North Carolina). In 1922, some fish from Spain were transferred to Lazio in Italy and from there throughout Italy and many countries including Germany, former Yugoslavia, Russia, Palestine and Rhodes (Sella 1926). Therefore, Hol1, the most frequent haplotype throughout Europe, likely corresponds to the first introduction, which originated from North Carolina.

Some individuals in two countries (France and Greece) corresponded to a different haplotype (Hol5), which we have not identified in our limited sampling from North America. This haplotype is phylogenetically much closer to Hol1 than to Hol7, the latter being abundant in the samples of Florida and illustrating the distinction between *G. holbrooki* from the Atlantic drainages and populations from Florida and the Gulf coast (Wooten et al. 1988; Scribner and Avise 1993). Although we have not yet found historical records, distribution of haplotype Hol5 is compatible with an independent introduction from the Atlantic drainages of North America and a restricted propagation through Europe. The fact that mosquitofish in Greece was introduced from Italy and France agrees with the presence of Hol1 and Hol5 haplotypes in Greek collections and suggests that Hol5 was originally introduced to France.

Reduced genetic diversity of European mosquitofish

We have demonstrated that *G. holbrooki* in Europe displays less genetic diversity than in its native American range. Founder events and population bottlenecks in early stages of introductions are considered responsible for the loss of diversity of many invasive species (Allendorf and Lundquist 2003; Roman and Darling 2007; Suarez and Tsutsui 2008; Dlugosch and Parker 2008) and should be expected in European mosquitofish because only 12 individuals were introduced in 1921 to Spain (Nájera Angulo 1944) and the following year 200 descendants from these were transferred to Italy (Artom 1924) and were thus the basis for the spread throughout Europe. Genetic diversity of *G. holbrooki* in Italy and Spain has already been shown to be low for nuclear markers as a consequence of the founder event during its introduction (Grapputo et al. 2006). In addition, haplotype diversity within native populations of *G. hoolbroki* was also reduced and potentially stressed the founder events in the European introduction. However, some European populations showed a higher amount of diversity than native American ones. This increased genetic diversity within populations has also been found in several other introduced species (Kolbe et al. 2004), resulting from a combination of multiple local introductions of several origins and numerous translocations from these sites of introduction (Roman and Darling 2007).

Another less understood source of increased genetic diversity in introduced locations are local mutations (Lee 2002). This may be the case of haplotype Hol3, which was restricted to heteroplasmic fish in the Millars locality. The nucleotide change distinguishing this Hol3 haplotype was the only one producing a non-conservative aminoacid substitution. To our knowledge, this is the first reported case of

heteroplasmy in the genus *Gambusia*. The presence of this polymorphism could be explained either by no negative effects on individual fitness or by small population size allowing the accumulation of deleterious mutations (the so called Muller's Ratchet effect). Predicted effects of this particular substitution with the PolyPhen tool (http://genetics.bwh.harvard.edu/pph) are benign, probably indicating that fitness is not affected. It is worth mentioning, however, that the actual effects of this haplotype could be masked because of the other heteroplasmic haplotype, Hol1.

In summary, our results show that introduced populations of invasive species often gain genetic diversity from multiple introductions and translocations (Facon et al. 2008). Local, "de novo" mutations could also play a role in *G. holbrooki*, a mechanism that needs further study in invasive introduced species.

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References

Alcaraz C, Bisazza A, García-Berthou E (2008) Salinity mediates the competitive interactions between invasive mosquitofish and an endangered fish. Oecologia 155:205–213

Allendorf FW, Lundquist LL (2003) Introduction: population biology, evolution, and control of invasive species. Conserv Biol 17:24–30

Anonymous (1927) Introduzione della "Gambusia patruelis" in Italia. Riv Malariol 6:999-1000

Artom C (1924) La specie di Gambusia acclimatata in Italia (*Gambusia holbrooki* Grd) in relazione colla stabilità del carattere del gonopodio. Atti Acc Naz Lincei 33:278–282

Bandelt H-J, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37–48

Benejam L, Alcaraz C, Sasal P, Simon-Levert G, García-Berthou E (2009) Life history and parasites of the invasive mosquitofish (*Gambusia holbrooki*) along a latitudinal gradient. Biol Invasions. doi:10.1007/s10530-008-9413-0

Congdon BC (1994) Salinity-related fitness differences amongst GPI genotypes in the mosquitofish *Gambusia holbrooki* (Poeciliidae: Teleostei). Biol J Linn Soc 53: 343–352

Congdon BC (1995) Unidirectional gene flow and maintenance of genetic diversity in the mosquitofish *Gambusia holbrooki* (Teleostei: Poeciliidae). Copeia 1995:162–172

Courtenay WR Jr, Meffe GK (1989) Small fishes in strange places: a review of introduced poeciliids. In: Meffe GK, Snelson FF (eds) Ecology and evolution of livebearing fishes (Poeciliidae). Englewood Cliffs, New Jersey, pp 319–331

Davis SK, Echelle AA, Van Den Bussche RA (2006) Lack of cytonuclear genetic introgression despite long-term hybridization and backcrossing between two poeciliid fishes (*Gambusia heterochir* and *G. affinis*). Copeia 2006: 351–359

Dlugosch KM, Parker IM (2008) Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. Mol Ecol 17:431–449

Ekmekçi FG, Kirankaya ŞG (2006) Distribution of an invasive fish species, *Pseudorasbora parva* (Temminck & Schlegel, 1846) in Turkey. Turk J Zool 30:329–334

Estoup A, Largiadier CR, Perrot E, Chourrout D (1996) Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes. Mol Mar Biol Biotechnol 5:295–298

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50

Facon B, Pointier JP, Jarne P, Sarda V, David P (2008) High genetic variance in life-history strategies within invasive populations by way of multiple introductions. Curr Biol 18:363–367

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

García-Berthou E, Alcaraz C, Pou-Rovira Q, Zamora L, Coenders G, Feo C (2005) Introduction pathways and establishment rates of invasive aquatic species in Europe. Can J Fish Aquat Sci 62:453–463

Grapputo A, Bisazza A, Pilastro A (2006) Invasion success despite reduction of genetic diversity in the European populations of eastern mosquitofish (*Gambusia holbrooki*). Ital J Zool 73:67–73

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Haynes JL, Cashner RC (1995) Life-history and population-dynamics of the western mosquitofish: a comparison of natural and introduced populations. J Fish Biol 46: 1026–1041

Hrbek T, Seckinger J, Meyer A (2007) A phylogenetic and biogeographic perspective on the evolution of poeciliid fishes. Mol Phylogenet Evol 43:986–998

Innal D, Erk'akan F (2006) Effects of exotic and translocated fish species in the inland waters of Turkey. Rev Fish Biol Fisheries 16:39–50

Kats LB, Ferrer RP (2003) Alien predators and amphibian declines: review of two decades of science and the transition to conservation. Divers Distrib 9:99–110

Kolbe JJ, Glor RE, Rodriguez SL, Lara AC, Larson A, Losos JB (2004) Genetic variation increases during biological invasion by a Cuban lizard. Nature 431:177–181

Krumholz LA (1948) Reproduction in the western mosquitofish, *Gambusia affinis affinis* (Baird & Girard), and its use in mosquito control. Ecol Monogr 18:1–43

Lee CE (2002) Evolutionary genetics of invasive species. Trends Ecol Evol 17:386–391

Lydeard C, Wooten MC, Meyer A (1994) Cytochrome b sequence variation and a molecular phylogeny of the livebearing fish genus *Gambusia* (Cyprinodontiformes: Poeciliidae). Can J Zool 73:213–227

Mulvey M, Newman MC, Chazal A, Keklak MM, Heagler MG, Hales LJ (1995) Genetic and demographic responses of mosquitofish (*Gambusia holbrooki* Girard 1859) populations stressed by mercury. Environ Toxicol Chem 14:1411–1418

Nájera Angulo L (1944) Sobre la identificación de la *Gambusia holbrookii*. Bol R Soc Esp Hist Nat Biol 42:51–55

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York, p 512

Pyke GH (2008) Plague minnow or mosquito fish? A review of the biology and impacts of introduced *Gambusia* species. Ann Rev Ecol Syst 39:171–191

Rauchenberger M (1989) Systematics and biogeography of the genus *Gambusia* (Cyprinodontiformes: Poeciliidae). Am Mus Novit 2951:1–74

Rice JA, Sax DF (2005) Testing fundamental evolutionary questions at large spatial and demographic scales: species invasions as an underappreciated tool. In: Sax DF, Stachowicz JJ, Gaines SD (eds) Species invasions: insights into ecology, evolution, and biogeography. Sinauer Associates, Sunderland, pp 291–308

Roman J, Darling JA (2007) Paradox lost: genetic diversity and the success of aquatic invasions. Trends Ecol Evol 22: 454–464

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sax DF, Brown JH (2000) The paradox of invasion. Global Ecol Biogeogr 9:363–371

Scribner KT (1993) Hybrid zone dynamics are influenced by genotype-specific variation in life-history traits: experimental evidence from hybridizing *Gambusia* species. Evolution 47:632–646

Scribner KT, Avise JC (1993) Cytonuclear genetic architecture in mosquitofish populations and the possible roles of introgressive hybridization. Mol Ecol 2:139–149

Scribner KT, Avise JC (1994) Population cage experiments with a vertebrate: the temporal demography and cytonuclear genetics of hybridization in Gambusia fishes. Evolution 48:155–171

Sella M (1926) Pesci larvifagi. Riv Malariol 5:504–507

Sella M (1929) Gambusia e verde Parigi nella lotta antimalarica a Rovigno e cenni sulla lotta in Istria. Riv Malariol 8:357–392

Stearns SC (1983) The genetic basis of differences in life-history traits among six populations of mosquitofish (*Gambusia affinis*) that shared ancestors in 1905. Evolution 37:618–627

Suarez AV, Tsutsui ND (2008) The evolutionary consequences of biological invasions. Mol Ecol 17:351–360

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10:512–526

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Veenvliet P (2007) Species identity of *Gambusia* (Pisces: Poeciliidae) introduced to Slovenia. Natura Sloveniae 9: 43–46

Walters DM, Freeman BJ (2000) Distribution of *Gambusia* (Poeciliidae) in a southeastern river system and the use of fin ray counts for species determination. Copeia 2000: 555–559

Wooten MC, Lydeard C (1990) Allozyme variation in a natural contact zone between *Gambusia affinis* and *Gambusia holbrooki*. Biochem Syst Ecol 18:169–173

Wooten MC, Scribner KT, Smith MH (1988) Genetic variability and systematics of *Gambusia* in the southeastern United States. Copeia 1988:283–289