

**Research Article****Mitochondrial DNA authenticates *Gambusia affinis* (Baird and Girard, 1853) as the invasive mosquitofish in Taiwan**Chia-Hao Chang<sup>1,2,\*</sup>, Yu-Chun Wang<sup>3</sup>, Dong-Chen Lee<sup>1</sup>, Hui-Chen Yang<sup>1</sup> and Shih-Hui Liu<sup>4,\*</sup><sup>1</sup>Department of Life Science, Tunghai University, No. 181, Sec. 4, Taiwan Blvd., Taichung City 40704, Taiwan<sup>2</sup>Center for Ecology and Environment, Tunghai University, No. 181, Sec. 4, Taiwan Blvd., Taichung City 40704, Taiwan<sup>3</sup>Planning and Information Division, Fisheries Research Institute, No.199, Hou-Ih Road, Keelung City 20246, Taiwan<sup>4</sup>Department of Biological Sciences, National Sun Yat-sen University, No. 70, Lien-Hai Road, Kaohsiung City 80424, TaiwanAuthor e-mails: [chiahao0928@gmail.com](mailto:chiahao0928@gmail.com) (CHC), [ycwang@mail.tfrin.gov.tw](mailto:ycwang@mail.tfrin.gov.tw) (YCW), [andrew0921972239@gmail.com](mailto:andrew0921972239@gmail.com) (DCL), [winney1998@gmail.com](mailto:winney1998@gmail.com) (HCY), [liushihhui@gmail.com](mailto:liushihhui@gmail.com) (SHL)*\*Corresponding authors*

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**OPEN ACCESS****Abstract**

Two mosquitofishes, the western, *Gambusia affinis* (Baird and Girard, 1853) and eastern, *G. holbrooki* Girard, 1859, species, were treated as a single lineage from 1882 to 1988. Here, we assess whether the mosquitofish introduced into Taiwan in 1911 for mosquito control really was *G. affinis*, as documented, or *G. holbrooki* or a combination of both. Using mitochondrial cytochrome *b* as a genetic marker, we sequenced 78 mosquitofish specimens sampled from around Taiwan and found only one haplotype. Our phylogenetic analyses support that only the western mosquitofish (*G. affinis*) has invaded Taiwan. However, since *G. holbrooki* is present in the Taiwanese aquarium market, future invasion by the eastern mosquitofish is very likely.

**Key words:** western mosquitofish, introduction, poeciliid, cytochrome *b*, phylogenetics**Introduction**

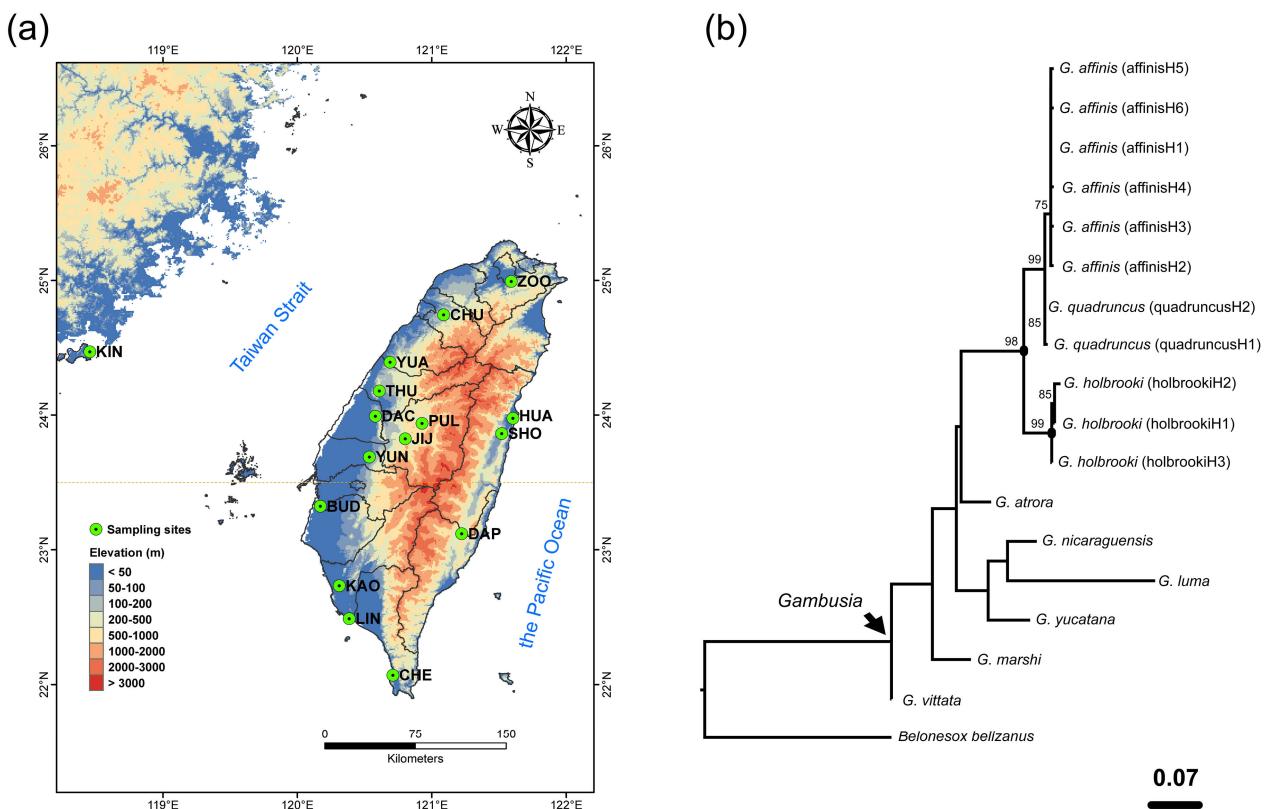
There is a long history of humans intentionally or accidentally transporting species to locations they would not be able to reach naturally. When such non-native species cause damage or are likely to be harmful to human or environmental health, they are called “invasive species” (Invasive Species Advisory Committee 2006). Human-mediated species dispersal has greatly increased in frequency and magnitude worldwide due to transport development (Crowl et al. 2008). Lowe et al. (2000) generated a list of the 100 worst invasive species in the world, five of which were fishes, including tilapia, Nile perch, rainbow trout, walking catfish, and mosquitofish.

The mosquitofish has been introduced in many localities to control mosquito populations and by association mosquito-borne diseases such as malaria (Pyke 2005). Compared to the other four worst invasive fishes listed by Lowe et al. (2000), the mosquitofish is not predatory and is much smaller, but it exhibits a broad tolerance to different environmental conditions and considerable plasticity in terms of behavior, morphology,

and trophic level (Stockwell and Weeks 1999; Pyke 2005; Ruehl and DeWitt 2005; Langerhans et al. 2007; Lee et al. 2018). Accordingly, the mosquitofish has successfully invaded a large territory, threatening indigenous animals on every continent except Antarctica (Pyke 2008).

Currently, two species of the invasive mosquitofish are recognized: the Western mosquitofish, *Gambusia affinis* (Baird and Girard, 1853) and the Eastern mosquitofish, *G. holbrooki* Girard, 1859. The taxonomic history of these two mosquitofishes is complicated since they are phylogenetically closely related and have similar morphologies (Lydeard et al. 1995; Walters and Freeman 2000). *Gambusia affinis* was described in 1853 based on specimens collected from two localities in Texas (Baird and Girard 1853). Later, *G. holbrooki* was described based on specimens collected from Charleston in South Carolina (Girard 1859). Subsequently, Jordan and Gilbert (1882) regarded these two fishes as a single species, *G. affinis*, but they continued to be viewed as two “varieties” (von Harleman 1916–1917). Hildebrand (1927) then classified them into two subspecies, *G. affinis affinis* and *G. affinis holbrooki*, which was supported by Black and Howell (1979). However, comprehensive molecular and morphological analyses demonstrated that there are two distinct species of mosquitofish, *G. affinis* and *G. holbrooki* (Wooten et al. 1988; Rauchenberger 1989). Introductions of mosquitofish worldwide for mosquito control began in the first half of the twentieth century (Francis 2012). At that time, *G. holbrooki* was a synonym of *G. affinis*, so introduction records referring to *G. affinis* may not be so precise. For example, records indicate that both *G. affinis* and *G. holbrooki* were introduced to Europe, but molecular analyses only detected *G. holbrooki* in Europe (Vidal et al. 2010), perhaps due to introgression between these two species or simple taxonomic confusion (Scribner and Avise 1994). Other molecular studies have also identified the species of mosquitofish invasive in Australia, Argentina, Greece, and Turkey as being *G. holbrooki* (Ayres et al. 2010; Triantafyllidis et al. 2011; Keskin et al. 2013; Cabrera et al. 2017), but it is *G. affinis* in mainland China, Malaysia, Myanmar, Java, Bali, and Saipan (James et al. 2010; Kano et al. 2016; Walton et al. 2016; Dahruddin et al. 2017; Gao et al. 2017).

Records show that the western mosquitofish (*G. affinis*) was introduced once into Taiwan in 1911, i.e. when Taiwan was a dependency of Japan, and it was then introduced to Japan from Taiwan in 1916 (Sato et al. 1972; Francis 2012). By the end of the 1920s, it was very common in Taiwan (Oshima 1920). Two DNA barcoding sequences published in the Fish Database of Taiwan (<http://fishdb.sinica.edu.tw/chi/species.php?id=381033>) support that *G. affinis* is the species occurring in Taiwan, but the eastern mosquitofish may have also invaded Taiwan through being mixed in the original western mosquitofish introduction stock or in later introduction events. Many poeciliid fishes have invaded Taiwan via the aquarium trade, such as *Poecilia reticulata* Peters, 1859, *P. velifera* (Regan, 1914), *Xiphophorus*



**Figure 1.** (a) Sampling localities of *Gambusia* specimens collected in Taiwan. (b) Maximum-likelihood tree of *Gambusia* inferred from mitochondrial cytochrome b sequences (305 base pairs) with 1000 bootstrap replicates. Each terminal is labeled with the scientific name and its haplotype code. Solid circles on branch nodes indicate statistically robust nodes with bootstrapping values in the ML tree  $\geq 70$ , and corresponding bootstrapping values  $\geq 70\%$  of the NJ tree are also marked on the branches.

*hellerii* Heckel, 1848, and *X. maculatus* (Günther, 1866) (Liang et al. 2006; Chen et al. 2010). Since both eastern and western mosquitofish are aquarium fishes, it is possible that both have invaded Taiwan, but *G. holbrooki* remains undocumented because both species are morphologically similar (Renard et al. 2000; Wu et al. 2011; Rao et al. 2015; Dufresnes et al. 2017). In this study, we hypothesized that, based on available records, *G. affinis* is the only invasive mosquitofish in Taiwan and tested our hypothesis by molecular authentication of specimens collected from around Taiwan.

## Materials and methods

Mosquitofish were collected using minnow traps and handnets from 24 September 2018 to 24 December 2018. A total of 78 specimens were gathered from 16 localities around Taiwan (Figure 1a, Supplementary material Table S1). At each locality, sampled specimens were euthanized with 0.025% MS-222, and then fixed in 95% ethanol. DNA samples were extracted from caudal fin tissue using a Genomic DNA Mini Kit (GT100, Geneaid, Taiwan). PCR amplifications of the 5' partial mitochondrial cytochrome *b* (*cyt b*) were performed in 20 ng template DNA, 12.5 µL of 2xTaq PCR MasterMix (Genomics, Taiwan), 10 µmol of each specific primer—Poeciliid *cyt b*-F (5'-GGATTAGAYGCTACYGCTACCA-3') and Poeciliid *cyt b*-R (5'-GRAAGTGGAAAGGCGAAGAACG-3'), which were

designed on the basis of the mitochondrial genomes of *Poecilia reticulata* (NC\_024238), *Xiphophorus hellerii* (FJ226476), *G. holbrooki* (NC\_028274), and *G. affinis* (NC\_004388)—made up to a final volume of 25 µL using distilled water. Thermal cycling began with one cycle at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec, and a final extension step at 72 °C for 10 min. PCR products were purified using a PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan). Approximately 50 ng of the purified PCR products was employed as template for sequencing, which was performed following the protocol of the ABI PRISM BigDye Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) using the Poeciliid-F primer. The 5' and 3' ends of the *cyt b* sequences were trimmed using the program CodonCode Aligner, and the final length of each *cyt b* sequence was 402 base pairs. After editing the 78 *cyt b* sequences, we only observed one haplotype, which has been submitted to GenBank with accession number MK370170.

We used the *cyt b* DNA sequences of *Belonesox belizanus* Kner, 1860 (JX556410), downloaded from GenBank, to serve as outgroups for our phylogenetic analysis (Hrbek et al. 2007; Reznick et al. 2017). To construct a reference database, we downloaded *cyt b* sequences from nine *Gambusia* fishes (Table S1). All *cyt b* sequences were aligned using the TranslatorX server (<http://www.translator.co.uk>), which is designed to align protein-coding nucleotide sequences based on their corresponding amino acid translations (Abascal et al. 2010). We employed two different phylogenetic approaches. A maximum-likelihood (ML) tree was constructed in RAxML 8.0.0 (Stamatakis 2014) from the alignment partitioned by codon position and using the GTR+G+I substitution model. The default algorithm of the software was used to perform 100 runs, and the best ML tree was chosen based on likelihood scores among suboptimal trees created by each run. Non-parametric bootstrap iterations ( $n = 1000$ ) provided nodal support. We also generated a neighbor-joining (NJ) tree based on Kimura two-parameter (K2P) distances with  $1 \times 10^7$  bootstrapping replicates in MEGA 7 software (Kumar et al. 2016).

## Results

Among the 78 sampled mosquitofish specimens, we only detected one haplotype, affinisH1, and this haplotype has also been found in specimens from Missouri, Mississippi, and Texas (USA), as well as mainland China (Table S1). A total of 305 base pairs were aligned for a combined dataset of 29 taxa, which contained 92 variable sites and 51 parsimony informative sites. Both our NJ and ML phylogenetic analyses support that *G. affinis*, *G. quadruncus* Langerhans, 2012, and *G. holbrooki* group together with high statistical support (bootstrapping value  $\geq 70$ ) (Figure 1b). However, only the NJ phylogenetic analysis clearly demonstrates that the haplotypes

from these three *Gambusia* fishes formed three distinct monophyletic groups, with affinisH1 and the other *G. affinis* haplotypes (affinisH2 to affinisH6) being clustered with high statistical support.

## Discussion

Although our dataset comprises only 305 base pairs of *cyt b*, the topology of our ML phylogenetic tree (Figure 1b) is highly concordant with those of other studies (Langerhans et al. 2012; Gao et al. 2017; Reznick et al. 2017). Our tree shows that the three *Gambusia* species—*G. affinis*, *G. quadruncus*, and *G. holbrooki*—are closely related. Since our Taiwanese mosquitofish specimens have the same haplotype as *G. affinis* sampled from mainland China and the United States, and as our phylogenetic analysis showed that the affinisH1 haplotype grouped with other *G. affinis* haplotypes, it is clear that only the western mosquitofish has invaded Taiwan. *Gambusia* fishes can hybridize (Davis et al. 2006; Echelle et al. 2013). Although *G. affinis* and *G. holbrooki* are also known to hybridize, Scribner and Avise (1994) demonstrated that *G. holbrooki* genotypes are more dominant than those of *G. affinis* upon hybridization, so the power of molecular identification to identify *G. affinis* in hybrid zones diminishes rapidly after just a few generations. Thus, even though we only employed a mitochondrial marker to detect *G. affinis* in Taiwan, we should still have been able to detect invasive *G. holbrooki* genotypes if hybridization had occurred between *G. holbrooki* and *G. affinis*.

*G. affinis* exhibits extremely low genetic diversity in Taiwan; only one *cyt b* haplotype was found from among 78 specimens sampled from around the island. The same outcome was observed for the *G. affinis* population on mainland China (Gao et al. 2017). This extremely low genetic diversity implies that the Taiwanese western mosquitofish population is derived from a single introduction event. However, how then can we explain the genetic paradox of its low genetic diversity (which should reduce its adaptive potential) yet successful invasion (Frankham 2004)? It remains to be established if the source population for *G. affinis* introductions was admixed (as observed for *Pseudorasbora parva* (Temminck and Schlegel, 1846), Simon et al. 2011), if the species' origin represents an “invasive bridgehead” that increased its adaptive potential (as reported for invasive *G. holbrooki* in Europe, Vera et al. 2016), or if genetic mechanisms such as transposable elements allow *G. affinis* to adapt quickly to new environments (Stapley et al. 2015). Further investigations of the population structure of western mosquitofish in its native and invasive distribution areas and the *G. affinis* genome (Hoffberg et al. 2018) could provide answers to these questions.

According to the records, *G. affinis* was introduced onto mainland China via two distinct invasion routes. One is that *G. affinis* was first introduced to Taiwan from North American, and then it was introduced to

mainland China from Taiwan. The other is that *G. affinis* was first introduced to Hawaii from Texas, from where it was transported to the Philippines and finally on to mainland China (Juliano 1989; Xie et al. 2001; Francis 2012). The first of these invasion routes perfectly explains why the western mosquitofish in Taiwan and mainland China share the same haplotype. However, by receiving *G. affinis* via two distinct routes, it might be expected that mainland China would display a more varied haplotype profile. There are three possible scenarios that explain this outcome.

First, Gao et al. (2017) only sequenced 33 specimens, so limited sampling may have precluded finding other *cyt b* haplotypes. Second, although mainland China received western mosquitofish from two distinct routes, the source populations may not have differed. Notably, the *affinisH1* haplotype is present in both Texas and Mississippi (Table S1), indicating that this haplotype is broadly distributed. Thus, even if the source populations for the two invasion routes differ, the *affinisH1* haplotype might have been present in both source populations. Third, random effects play a major role in the population genetics of invasive species, but we cannot rule out the impact of selection. Certain mitochondrial haplotypes could be adaptively advantageous during introductions. For example, Chien et al. (2016) demonstrated that some mitochondrial lineages in grey mullet (*Mugil cephalus*) developed larger ovaries. Consequently, if the *affinisH1* haplotype confers some characters, such as a higher growth rate or more offspring, on western mosquitofish, we would expect that this haplotype would ultimately predominate in the invasive population.

Finally, our molecular data as well as records in the literature demonstrate that only *G. affinis* was introduced into Taiwan. Although both *G. affinis* and *G. holbrooki* are aggressive invasive species, clear evidence to support that they are sympatric in areas other than the United States has not yet been reported (Wooten and Lydeard 1990; Walters and Freeman 2000). This scenario may simply be due to the fact that these two fishes were separately introduced to distinct areas or interspecific competition drives one of the species to extinction where they encounter each other. For example, Scribner (1993) demonstrated that the population size of *G. affinis* was suppressed where it was sympatric with *G. holbrooki*. In October 2018, *G. holbrooki* became available in the Taiwanese aquarium market (<https://fishbook.com.tw/2018%E5%B9%B410%E6%9C%88%E6%96%B0%E9%AD%9A%E5%BF%AB%E8%A8%8A/>), greatly increasing the risk of *G. holbrooki* invasion. Continuous monitoring of wild mosquitofish populations in Taiwan based on both mitochondrial and nuclear gene markers would not only provide an early alert to *G. holbrooki* invasion and hybridization with *G. affinis*, but also facilitate research into interspecific competition between these two fishes in an invasion area.

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#### Supplementary material

The following supplementary material is available for this article:

**Table S1.** Cytochrome *b* (*cyt b*) sequences from poeciliid fishes used for ML and NJ phylogenetic analyses.

This material is available as part of online article from:

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