

Research Article

Molecular and morphometric evidence for the widespread introduction of Western mosquitofish *Gambusia affinis* (Baird and Girard, 1853) into freshwaters of mainland China

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Abstract

Two North American species of mosquitofish, the Western (*Gambusia affinis* Baird and Girard, 1853) and Eastern mosquitofish (*G. holbrooki* Girard, 1859), rank amongst the most invasive freshwater fishes worldwide. While the existing literature suggests that *G. affinis* was introduced to mainland China, empirical evidence supporting this assumption was limited, and the possibility remained that both species were introduced during campaigns attempting to reduce vectors of malaria and dengue fever. We used combined molecular information (based on phylogenetic analyses of sequence variation of the mitochondrial cytochrome *b* gene) and morphometric data (dorsal and anal fin ray counts) to confirm the presence of only one species, *G. affinis*, across ten sampling sites in mainland China.

Key words: aquatic invasions, freshwater fish, plague minnow, phylogenetic analyses, species identification

Introduction

The Eastern mosquitofish *Gambusia holbrooki* Girard, 1859 is widely distributed in freshwaters along the East coast of the United States of America, while the mid-western parts of the United States and North-eastern Mexico are inhabited by its morphologically very similar sister species, the Western mosquitofish *Gambusia affinis* (Baird and Girard, 1853) (Jordan et al. 1930; Pyke 2008). Nowadays, both species—often referred to as plague minnows—rank amongst the top 100 most invasive species worldwide (Lowe et al. 2000). In most cases, mosquitofish were deliberately introduced into freshwater bodies to control mosquito populations and the diseases they transmit (especially malaria and dengue fever; Pyke 2005).

As small and fast-growing, livebearing (ovoviviparous) fishes, both species have high reproductive potential (Milton and Arthington 1983; Trendall 1982;

Turner 1937; Vargas and de Sostoa 1996) and tolerance to a broad range of environmental conditions (e.g., salinities ranging from 0–41 ppt, and dissolved oxygen ranging from 1–11 mg L⁻¹; Pyke 2008; Uliano et al. 2010). Mosquitofish prey on various small aquatic organisms so introductions can cause declines of native fish, amphibian, and invertebrate populations (Pyke 2008; Srean 2015). For example, the reduction of Sonoran topminnow (*Poeciliopsis occidentalis* Baird and Girard, 1853) populations throughout much of their native range is due to predation on juvenile topminnows by invasive *G. affinis* (Meffe 1985). Likewise, declining abundances of *Litoria aurea* Lesson, 1831 frogs in water-bodies of southeastern Australia can be attributed to predation on tadpoles by invasive *G. holbrooki* (Hamer et al. 2002; Morgan and Buttemer 1996).

While the documentation of mosquitofish introductions is incomplete, several routes of introductions have

Figure 1. Sites in mainland China from which mosquitofish were reported, including published records (sites A–K, represented by triangles): A. Suzhou; B. Shanghai; C. Fuzhou; D. Xiamen; E. Guangzhou; F. Haikou; G. Nanning; H. Kunming; I. Chongqing; J. Wuhan; K. Hengyang. Circles (sites 1–10) denote sampling sites from which fish were collected for our present study: 1. Baoding; 2. Nangjing; 3. Huzhou; 4. Hangzhou; 5. Lishui; 6. Quanzhou; 7. Xiamen; 8. Beihai; 9. Chengdu; 10. Ankang (for details see Supplementary material Table S1).



been documented. For example, one route involved the introduction of *G. affinis* from Illinois (USA) to Trieste (Italy) in 1927 (Krumholz 1948). Another route involved the introduction of *G. holbrooki* from North Carolina to Spain (Artom 1924; Nájera Angulo 1944; Vidal et al. 2010), then to Italy and on to other locations in Europe and Australia (Krumholz 1948; Pyke 2008). Yet another route involved the introduction of *G. affinis* from Texas to Hawaii and from there to other Pacific islands, including New Zealand (Jordan et al. 1930), the Philippines (Juliano et al. 1989) and Taiwan (Liao and Liu 1989).

Screening the available (international and local, i.e. Chinese) literature revealed two records of mosquitofish introductions to mainland China. One reports that mosquitofish were introduced to mainland China (no location information provided) from Taiwan in 1924 (Xie et al. 2001; Xu and Qiang 2004), while the second record reports on an introduction into freshwaters in Shanghai from Manila, Philippines in 1927 (Krumholz 1948; Li and Xie 2002; Li et al. 2007; Tan and Tong 1989). Therefore, invasive mosquitofish in mainland China are likely *G. affinis*. However, reports of mosquitofish introductions to either Taiwan or the Philippine Islands date back to the early 1920s (Juliano et al. 1989; Seale 1917; Xie et al. 2001), when the taxonomic distinctiveness of *G. affinis* and *G. holbrooki* was not yet established (both taxa were thought to be subspecies of *G. affinis*

at that time). Only in the late 1980s were *G. affinis* and *G. holbrooki* recognized as two separate species (Wooten et al. 1988). Therefore, the possibility remains that invasive mosquitofish in the Philippines are actually *G. holbrooki*, or that both species were co-introduced. To further illustrate the confusion stemming from an uncertain taxonomic status of both species, invasive mosquitofish in Europe have long been considered to be *G. affinis* (Krumholz 1948), while later studies demonstrated the presence of *G. holbrooki* throughout southern Europe (Vidal et al. 2010). Likewise, invasive mosquito-fish in Lake Nainital in India were previously identified as *G. affinis* but are actually *G. holbrooki* (Singh and Gupta 2007). For most Asian countries from which *G. affinis* have been reported, molecular information is missing, a notable exception being a study on mosquitofish in Lake Kenyir, Malaysia, where presence of *G. affinis* was verified using DNA barcoding (Walton et al. 2016).

To date, mosquitofish are widely distributed in mainland China (Figure 1, Supplementary material Table S1); however, it remains unclear if *G. affinis*, *G. holbrooki*, or both species were introduced. In this study, we used combined morphometric information (fin ray counts; Walters and Freeman 2000) and sequence variation of the mitochondrial cytochrome *b* gene to determine the species identity of *Gambusia* populations in mainland China.

Material and methods

Biological material and meristic morphology (fin rays counts)

We collected mosquitofish from 10 sampling sites in mainland China between April and September 2015 using dip nets (3 mm mesh size). Our sampling scheme roughly followed published records on the occurrence of mosquitofish (Figure 1). Sample sizes ranged from $n = 63$ to $n = 94$ adult fish per site. All fish were sacrificed immediately upon capture and preserved in 96% ethanol for later examination in the laboratory.

A subsample of $n = 200$ individuals (10 randomly selected female and 10 male fish per site) was used to collect information on numbers of anal and dorsal fin rays. For comparison, we also included two populations of *G. holbrooki* into our assessment of fin ray numbers (one aquarium-reared stock of Floridian origin and one field-collected sample originating from Comacchio, Italy, where the species is invasive; Vidal et al. 2010). Sample sizes were $n = 10$ (5 female and 5 male) *G. holbrooki* per population.

The skin layer covering fin rays renders direct inspection of fin rays under the stereomicroscope a challenging task and so we first digested soft tissues (e.g., Rawson et al. 2006). Specifically, non-calcified tissues were removed by immersing the whole fish in 1% potassium hydroxide (KOH) for five days. We counted dorsal and anal fin rays on photographs that were taken using a high-performance Full HD HDMI camera (OPTIKA, Ponteranica, Italy) mounted on a stereomicroscope (szn-6 OPTIKA, Ponteranica, Italy).

We used Binomial tests to compare frequency distributions of dorsal and anal fin ray counts against the expected values published for *G. holbrooki* (i.e., 8 dorsal and 11 anal fin rays; Walters and Freeman 2000). Hence, significant results would indicate presence of *G. affinis* but not *G. holbrooki* in our samples from mainland China, while inclusion of two confirmed *G. holbrooki* populations served as a control to verify our methodological approach.

Another approach would have been to investigate the morphology of the male copulatory organ, the gonopodium, as structures and contours of the gonopodium tip tend to differ between *G. affinis* and *G. holbrooki* (Rauchenberger 1989). We decided not to use this approach because 1) analyses of gonopodium morphology fail to identify female individuals and 2) ecological variation—especially differences in predation pressure—are known to generate intra-specific diversity in gonopodium morphology (Heinen-Kay and Langerhans 2013; Heinen-Kay et al. 2014). Using landscape morphometrics, we confirmed

pronounced variation in gonopodium morphology among *G. affinis* populations across mainland China (Ouyang et al., unpublished data).

Phylogenetic analyses

We extracted whole genomic DNA from pectoral fin tissue using the EasyPure Genomic DNA Kit (Beijing TransGen Biotech, Beijing, China). In most cases, we included $n = 3$ specimens per population. However, in the case of the Chengdu population, where some variability in dorsal fin ray counts was detected (see Results), we included $n = 6$ individuals ($n = 3$ with seven dorsal fin rays and $n = 3$ with eight dorsal fin rays). We used the primers CytBF1 (5'-ATG GCC AAC CTA CGA AAA AC-3') and CytBR1 (5'-GGG TAG RAC ATA ACC TAC GAA G-3'; Vidal et al. 2010) to amplify a fragment of the mitochondrial cytochrome *b* gene. Cycle sequencing was carried out in a final volume of 50 μ L, containing 2 mM MgSO₄, 0.2 mM dNTPs, 2.5 units EasyTaq DNA Polymerase (Beijing TransGen Biotech, Beijing, China), 0.2 μ M of each primer, and 25 ng of genomic DNA. PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s (denaturation), 60 °C for 30 s (primer annealing), 72 °C for 30 s (elongation), and a final elongation step at 72 °C for 10 min. PCR products were isolated via gel-electrophoresis and purified with the EasyPure Quick Gel Extraction Kit (Beijing TransGen Biotech, Beijing, China) and then sequenced by Genscript Corporation (Nanjing, China) using the BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA, USA) and the CytBF1 primer.

Sequence alignments were performed with ClustalW using default parameters and further analyses were restricted to the aligned fragment of 307 bp length. Phylogenetic analyses were conducted using MEGA5 (Tamura et al. 2011) after assessing potential substitution saturation with DAMBE (Xia et al. 2003). We constructed a neighbor-joining (NJ) tree using 1,000 bootstrap replicates under a p-distance model. We also calculated Bayesian posterior probabilities values (*PP*) using MrBayes 3.1.2 while applying a HKY+ Γ model (i.e., the best model of sequence evolution identified using Jmodeltest, Darriba et al. 2012). MrBayes was run for 1000,000 generations, sampling trees every 100 generations, with the initial 25% of trees discarded as burn-in (after checking that stationarity was reached).

Besides sequences generated from Chinese mosquitofish in the course of this study, we included 20 additional partial cytochrome *b* sequences obtained from GenBank (Table 1), including different *Gambusia* species (*G. geiseri*, *G. speciosa*, *G. krumholzi*, *G.*

Table 1. Information of cytochrome *b* sequences from other members of the genus *Gambusia* and additional sequences of *G. affinis* and *G. holbrooki* obtained from GenBank.

| Species | GenBank ID | Source | References |
|-----------------------|------------|--|------------------------|
| <i>G. geiseri</i> | U18207.1 | San Marcos, Hays County, Texas, USA | Lydeard et al. 1995 |
| <i>G. speciosa</i> | JX679671.1 | San Felipe Creek, Texas, USA | Echelle et al. 2013 |
| <i>G. krumholzi</i> | JX679663.1 | Coahuila, Mexico | Echelle et al. 2013 |
| <i>G. clarkhubbsi</i> | JX275483.1 | Del Rio, San Felipe Creek, Texas, USA | Unpublished |
| <i>G. heterochir</i> | U18208.1 | Clear Creek, Menard County, Texas, USA | Lydeard et al. 1995 |
| <i>B. belizanus</i> | JX556410.1 | Río Grande, Belize | Unpublished |
| <i>G. affinis</i> 1 | EF017514.1 | Merrimack River, Missouri, USA | Hrbek et al. 2007 |
| <i>G. affinis</i> 2 | DQ075683.1 | Clear Creek, Menard County, Texas, USA | Davis et al. 2006 |
| <i>G. affinis</i> 3 | DQ075681.1 | Clear Creek, Menard County, Texas, USA | Davis et al. 2006 |
| <i>G. affinis</i> 4 | HM443905.1 | Las Moras Creek, Maverick County, Texas, USA | Langerhans et al. 2012 |
| <i>G. affinis</i> 5 | U18107.1 | Village Creek, Hardin County, Texas, USA | Lydeard et al. 1995 |
| <i>G. affinis</i> 6 | DQ075686.1 | Clear Creek, Menard County, Texas, USA | Davis et al. 2006 |
| <i>G. affinis</i> 7 | KF013227.1 | San Francisco Estuary-Delta, USA | Brandl et al. 2015 |
| <i>G. affinis</i> 8 | HQ609579.1 | Big Black River, Mississippi, USA | Vidal et al. 2010 |
| <i>G. affinis</i> 9 | HQ609580.1 | San Saba River, Texas, USA | Vidal et al. 2010 |
| <i>G. affinis</i> 10 | HQ609581.1 | San Saba River, Texas, USA | Vidal et al. 2010 |
| <i>G. affinis</i> 11 | HQ609590.1 | Big Black River, Mississippi, USA | Vidal et al. 2010 |
| <i>G. holbrooki</i> 1 | U18210.1 | Highway A1A, Key West, Florida, USA | Lydeard et al. 1995 |
| <i>G. holbrooki</i> 2 | HM443916.1 | Miami-Dade Everglades, Florida, USA | Langerhans et al. 2012 |
| <i>G. holbrooki</i> 3 | KP723574.1 | Vrbnik pond, Krk, Croatia | Unpublished |
| <i>G. holbrooki</i> 4 | HM443917.1 | Branches Hammock, Florida, USA | Langerhans et al. 2012 |
| <i>G. holbrooki</i> 5 | HM443915.1 | Key West, Florida, USA | Langerhans et al. 2012 |
| <i>G. holbrooki</i> 6 | HM443918.1 | Sayre River, South Carolina, USA | Langerhans et al. 2012 |
| <i>G. holbrooki</i> 7 | JN565048.1 | Currambeen Creek, NSW, Australia | Unpublished |

clarkhubbsi, *G. heterochir*). We included 11 additional sequences of *G. affinis* (*G. affinis* 1 – *G. affinis* 11) and six additional sequences of *G. holbrooki* (*G. holbrooki* 1 – *G. holbrooki* 6) in an attempt to capture some of the genetic variation in both species' natural distribution ranges. We further included one outgroup species from the basal poeciliid tribe Gambusini (Bisazza et al. 1997; Mojica and Barlow 1997), namely the pike livebearer, *Belonesox belizanus* from Río Grande, Belize (JX556410.1; Table 1).

Results

Fin ray counts

Most of the $n = 200$ mosquitofish from ten sampling sites in mainland China had seven dorsal fin rays (Figure 2A) except for some fish collected in the city of Chengdu in Sichuan Province. Five (three males and two females) out of 20 fish in this population showed eight dorsal fin rays (Figure 2B). All mosquitofish collected in China had ten anal fin rays (Figures 2C and D). By contrast, all individuals of *G. holbrooki* included in this analysis showed eight dorsal and 11 anal fin rays (Supplementary material Figure S1). Hence, our data support the idea that *G. affinis*, but not *G. holbrooki*, has a wide invasive range of occurrence across mainland China (see Table 2 for details).

Phylogenetic relationships

All $n = 33$ individuals from which we obtained partial cytochrome *b* sequences had the same nucleotide sequence. This also applies to the $n = 3$ fish from Chengdu whose species identity based on dorsal fin ray counts was ambiguous (see above). We, therefore, treated our samples from mainland China as one sample ("Chinese samples" in Figure 3).

Our phylogenetic analyses found Chinese samples to cluster within the variation seen among *G. affinis* samples obtained from Genbank (*G. affinis* 1 through *G. affinis* 11); the monophyletic *G. affinis* clade received high bootstrap support (100) and a high posterior probability ($PP = 0.81$; Figure 3). A clade including all sequences of *G. holbrooki* (*G. holbrooki* 1 through *holbrooki* 6) also received high bootstrap support (99) and a high PP value (0.91), supporting the reciprocal monophyly of both species. In accordance with previous studies (Lydeard et al. 1995; Riesch et al. 2014; Vidal et al. 2010), we found *G. affinis* and *G. holbrooki* to be sister species (bootstrap value = 76, PP value = 0.79). All other species of *Gambusia* (*G. geiseri*, *G. speciosa*, *G. krumholzi*, *G. clarkhubbsi*, *G. heterochir*) were assigned to another cluster with high bootstrap support (97) and a high PP value (1.00).

Table 2. Species identification based on fin ray counts. Empirical dorsal fin rays counts were compared to the expected value reported for *G. holbrooki* (i.e., 8 fin rays versus 7 fin rays in *G. affinis*; Walters and Freeman 2000). Likewise, anal fin ray counts were compared to the expected value for *G. holbrooki* (i.e., 11 fin rays versus 10 fin rays in *G. affinis*; Walters and Freeman 2000). Results from Binomial tests for $n = 20$ fish per population (10 males, 10 females) are shown for the ten mosquitofish populations collected in mainland China, as well as data from an aquarium stock of *G. holbrooki* from Florida and an invasive population of *G. holbrooki* from Comacchio, Italy.

| Presumed species identity | Sampling site | number of individuals with dorsal fin rays counts | | | <i>P</i> -value | number of individuals with anal fin rays counts | | <i>P</i> -value |
|---------------------------|---------------|---|----|----------|-----------------|---|----------|-----------------|
| | | 7 | 8 | | | 10 | 11 | |
| <i>G. affinis</i> | Ankang | 20 | 0 | < 0.0001 | 20 | 0 | < 0.0001 | |
| | Baoding | 20 | 0 | < 0.0001 | 20 | 0 | < 0.0001 | |
| | Nangjing | 20 | 0 | < 0.0001 | 20 | 0 | < 0.0001 | |
| | Hangzhou | 20 | 0 | < 0.0001 | 20 | 0 | < 0.0001 | |
| | Huzhou | 20 | 0 | < 0.0001 | 20 | 0 | < 0.0001 | |
| | Quanzhou | 20 | 0 | < 0.0001 | 20 | 0 | < 0.0001 | |
| | Xiamen | 20 | 0 | < 0.0001 | 20 | 0 | < 0.0001 | |
| | Chaozhou | 20 | 0 | < 0.0001 | 20 | 0 | < 0.0001 | |
| | Beihai | 20 | 0 | < 0.0001 | 20 | 0 | < 0.0001 | |
| | Chengdu | 15 | 5 | 0.041 | 20 | 0 | < 0.0001 | |
| Total | | 195 | 5 | < 0.0001 | 200 | 0 | < 0.0001 | |
| <i>G. holbrooki</i> | Florida | 0 | 10 | 0.99 | 0 | 10 | 0.99 | |
| | Comacchio | 0 | 10 | 0.99 | 0 | 10 | 0.99 | |
| | Total | 0 | 20 | 0.99 | 0 | 20 | 0.99 | |

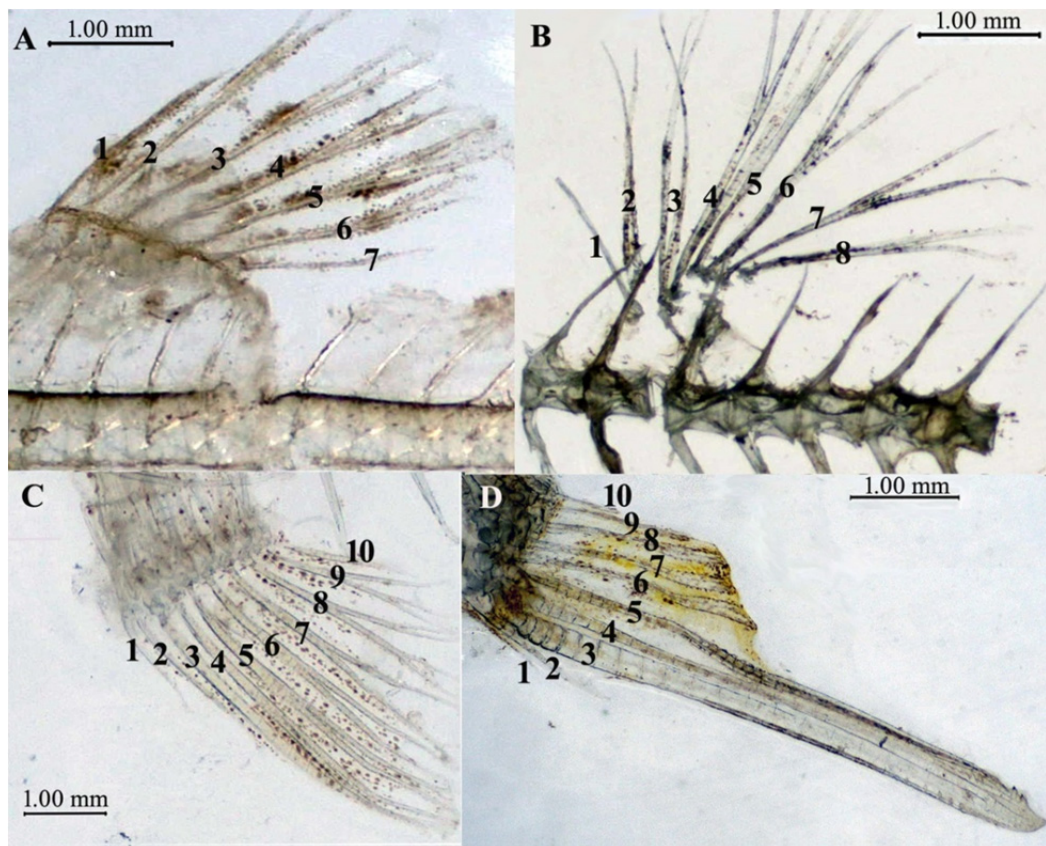


Figure 2. Exemplary photographs used to count dorsal and anal fin rays of *Gambusia affinis* in mainland China. Samples were treated with potassium hydroxide solution to remove soft tissues. (A) example of seven dorsal fin rays found in the population from Ankang, Shaanxi Province (site number 10); (B) example of eight dorsal fin rays found in Chengdu, Sichuan Province (site number 9); (C) anal fin ray count in a female from Ankang; (D) anal fin ray count in a male from Hangzhou, Zhejiang Province (site number 4). Anal fin rays no. 3, 4 and 5 form the copulatory organ, the gonopodium, in males (photographs by Jiancao Gao).

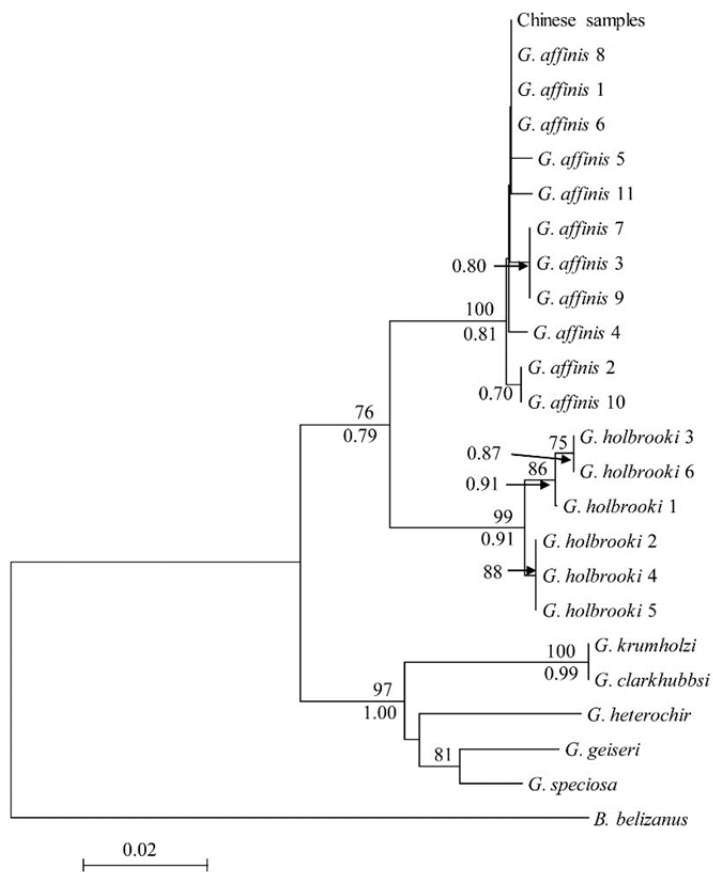


Figure 3. Phylogenetic analysis of representative *Gambusia* spp. based on partial cytochrome *b* sequences. Shown is a neighbor-joining tree along with bootstrap support for nodes under a p-distance model (only bootstrap support > 70 is shown). Bayesian posterior probability values > 0.70 are presented under the bootstrap values. Analysis of Chinese samples yielded a single nucleotide sequence, i.e., no variation among $n = 33$ individuals from ten sampling sites.

Discussion

In this study meristic morphometric information (fin ray numbers) and phylogenetic analyses based on sequence variation of the mitochondrial cytochrome *b* gene indicate that invasive mosquitofish in mainland China are Western mosquitofish, *G. affinis*. In line with the results of the study by Walters and Freeman (2000), all *G. affinis* from Chinese sampling sites had fewer anal fin rays than *G. holbrooki*. However, in our present study, some *G. affinis* with eight (not seven) dorsal fin rays were found in the population collected in Chengdu. Ambiguity of dorsal fin ray numbers in *G. affinis* (seven or eight rays) was also described by Hubbs (1926). Dorsal and anal fin ray numbers can be affected by fluctuation in ecological factors like temperature or salinity that fish experience during their embryonic development (Heuts 1949; Seymour 1959). We can only speculate that some individuals with eight dorsal fin rays in the Chengdu population are the result of unusually high temperatures at an early point of their development.

This could be brought about by the shallowness of their particular habitat—a pond in an urban park. Nevertheless, our study found all individuals from China to differ from *G. holbrooki* in numbers of anal fin rays, thus highlighting the usefulness of our meristic analysis for species discrimination.

All studied specimens from Chinese sampling sites yielded the exact same sequence of the 307 bp cytochrome *b* fragment. Based on the low degree of genetic variability, all mosquitofish in mainland China may have been introduced from the same source population, but future analyses using more markers (including fast-evolving nuclear markers like microsatellites), coupled with powerful population genetic approaches are needed to shed additional light on this question (see Sanz et al. 2013, for European *G. holbrooki*; Ayres et al. 2013, for Australian *G. holbrooki* and Purcell and Stockwell 2015, for *G. affinis* in New Zealand).

We currently have an incomplete understanding of the relative contributions of deliberate introductions across mainland China (i.e., man-made dispersal) in campaigns to reduce vectors of malaria (*Anopheles*

mosquitoes) and dengue fever (*Aedes* mosquitoes) as well as active dispersal along watersheds. In mainland China, invasive mosquitofish were first reported from Shanghai in 1927 (Li and Xie 2002; Zhang et al. 2013) and were used for deliberate introductions in that area (Pan et al. 1980), while in urban Guangzhou and surrounding areas mosquitofish were not reported until the 1960s (Pan et al. 1980). We hypothesize that Shanghai was the initial introduction site, and subsequent introductions, along with the (much slower) active dispersal of *G. affinis* along the Yangtze River and Pearl River drainages could explain the ubiquitous occurrence of *G. affinis* in Southern China.

Mosquitofish introductions continue to impose a massive threat not only to the conservation of indigenous biodiversity but also to fisheries (Arthington and Marshall 1999; Rowe et al. 2007). Where mosquitofish became invasive, they tend to cause decreases in populations of native aquatic species, including different detritivores, zooplankters (rotifers, crustaceans, backswimmers etc.), amphibians and fishes, all of which have the potential to affect vital components of ecosystem functioning (Blanco et al. 2004; Caiola and de Sostoa 2005; Hurlbert and Mulla 1981; Hurlbert et al. 1972; Margaritora et al. 2001; Mills et al. 2004; Rinco et al. 2002). Even the introduction of only few individuals bears risk of local population establishment and further range expansion, as mosquitofish have a high reproductive potential (Milton and Arthington 1983; Trendall 1982; Turner 1937; Vargas and de Sostoa 1996). It remains to be studied how aquatic faunas in freshwater ecosystems will change throughout mainland China, as the further spread of mosquitofish is likely only hampered by colder climates towards higher latitudes (Riesch et al. unpubl. data for *G. holbrooki*).

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Supplementary material

The following supplementary material is available for this article:

Table S1. Sites in mainland China from which *Gambusia affinis* were reported.

Appendix 1. References for Table S1.

Figure S1. Exemplary photographs of dorsal and anal fins of *Gambusia holbrooki* from Comacchio, Italy and Florida, USA.

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