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New data on the taxonomic status and distribution of *Gambusia sp.* in Croatia and Bosnia and Herzegovina

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Abstract

Background and Purpose: *In the early 20th century, two native North American poeciliid species Gambusia holbrooki and Gambusia affinis were introduced to Europe as a mosquito control agent. The first introduction to Istria from Italy in 1924 was followed by several independent introductions and massive translocations. Presently, the distribution of these two species in Croatia and Bosnia and Herzegovina (Bosnia and Herzegovina) is still largely unknown and they are often confused. The purpose of this study was to determine the taxonomic status of individual Gambusia populations and to shed more light on the distribution and phylogeographic patterns of these invasive species in Croatia and Bosnia and Herzegovina.*

Materials and Methods: *All new and literature data were plotted together to obtain the overall distribution of Gambusia sp. in Croatia and Bosnia and Herzegovina. The samples collected across this range were analysed meristically and by means of mitochondrial cytochrome b (cytb) gene sequence analysis to ascertain the true taxonomic status of the populations. For phylogeographic analysis, the cytb sequences from this study were combined with previously published data.*

Results and Conclusions: *The methods only confirmed the presence of G. holbrooki. G. holbrooki is continuously distributed across the entire Mediterranean biogeographical region in Croatia and Bosnia and Herzegovina. Two different mitochondrial cytb haplotypes were found: the widely spread Hol1 haplotype and haplotype Hol5 that in Europe was previously found only in France and Greece. This suggests that G. holbrooki populations in the region originated from different stocks.*

INTRODUCTION

Mosquitofish, *Gambusia sp*., freshwater fish species native to and widely distributed along the Gulf and Atlantic coasts of the United States are currently assigned to two distinct species: *Gambusia affinis* and *G. holbrooki* (*Gambusia*, Poeciliidae). In the course of mosquito and mosquito-borne diseases control efforts they were introduced throughout the world. However, beyond their native range they act as invasive species representing a threat to native biota *(1).* Their taxonomy has undergone a number of changes in the past including species synonymisation and elevation *(2).* Briefly, in the mid-19th century, three species were recognised: *G. affinis, G. patruelis* and *G. holbrooki*. Shortly after they were considered as a single polytypic species with two subspecies, western one, *G. affinis affinis* (encompassing former species *G. affinis* and *G. patruelis*) and eastern subspecies *G. affinis holbrooki*. However, their taxonomic status continued to be questioned. Black and Howell 1979 *(3)* reported about the existence of reproductive barriers between them, and genetic study of Wooten et al. 1988 *(4)* based on allozyme analysis, revealed a high degree of genetic divergence as well as the geographical structuring of allele frequencies. Taking all this into consideration, Wooten et al. 1988 *(4)* have elevated the two subspecies of *G. affinis*, to a species level. These two species have been successfully introduced from North America to most of the warmer parts of the world *(2)*. The distribution of these two species in the areas of introduction is still largely unknown and they are often confused primarily while the most of introductions took place before the last changes in their taxonomy, but also due to the lack or deficiency of historical records about the origins of introduced stocks as well as the complex patterns of their translocation *(2, 5)*. The same is true for Croatia and Bosnia and Herzegovina where, until recently, only *G. affinis* was listed at the official lists of invasive species. Detail analysis, of taxonomic status of *Gambusia sp.* present in this region, was never conducted.

In Croatian biological and medical literature *(6, 7, 8, 9, 10)*, we find different information on the year of introduction, the type of introduced mosquitofish and the extent of the initial introduction. These data mainly refer to the type of *G. affinis*, and the period from 1924 to 1932. Prof. Dr. Massimo Sella, biologist, researcher and former director of Office of malaria in Geneva, introduced the first mosquitofishes in Europe. In the 1919/1920 he obtained permission from the Fishery Board USA and the American Red Cross to send mosquitofishes to Italy and Spain, but these fish all died en route. In 1921, the US Agency Fishery Board reiterated the shipment, this time only in Spain (*G. holbrooki*, from Virginia). Fishes were released into the Estramadure area in antimalarial fight. In a few months, the fishes multiplied rapidly and in the 1922 Ettore Bora, officer of the Association for the Rehabilitation of wetlands, transferred mosquitofishes to Italy. Mosquitofishes (*G. holbrooki*) were planted in some ponds near the village Colonia Elena.

All Gambusia introduced in Italy belong to the species *G. holbrooki* (personal collection of AS / DAST 189-1), although some Italian literature indicates the wrong type as *G. affinis*. The Italians in „Notizie" from 1927 inform the quote „Initially, we thought that the imported species was *G. affinis*; but it turned that it was *G. holbrooki*" *(11)*. This professional misconception has persisted to the present day *(12)*.

Gambusia was introduced in Istria in 1924 from Italy by Gosia and Missirolia from the Directorate General of Public Health, and was planted into a swamp near Poreč for breeding, where in a few months they spread rapidly *(12)*. From this swamp, *Gambusia* were released in 202 aquatic collection points in the area of Poreč, Rovinj, Pula *(13)* and on island Krk *(14)* since they were well adapted to it. Mosquitofish passed through extremely cold winter of 1924/25, with temperatures as low as –12°C, despite expert opinions that Gambusia cannot stand northern winters *(12)*. In 1925 from Poreč, Gambusia was introduced to Koper (Slovenia) into a salty bay and canal, with salinity between 25–50‰. From Italy, mosquitofishes were dispatched to many countries: Germany, Russia, Yugoslavia and Palestine. In Italy they were introduced to all locations with malaria. *G. holbrooki* were significantly spread in many countries on the European continent. In 1926 Dr. Sella conducted another intake of mosquitofish from New York. Ship „President Wilson" had brought in Trieste 12 consignments with specimens of *G. patruelis* (*affinis*), significantly more resistant to cold *(15)*. The samples came from hatchery in Illinois (Urbana). These specimens were introduced into a pond near Rovinj and were multiplied during the spring of 1926. This gives clear indication that *G. affinis* was independently introduced in Croatia just two years after the introduction of *G. holbrooki 1924*. Kottlet and Freyhof 2007 *(16)* even question whether *G. affinis* was ever introduced in Europe, because they had no literature evidence of this event. Dr. Sella work clearly indicate that it was introduced in Istria in 1926, but the big question is did it survive or was over competed by *G. holbrooki*.

In August 1924, Dr. Trausmillera, director of Bacteriological station in Kraljevica, brought approximately 400 individuals of mosquitofish (*G. holbrooki*) on the island of Krk from Istria (Poreč). Mosquitofish are planted in several ponds on the island and in the spring of 1925 *Gambusia* were moved to 25 new ponds *(12)*. Dr. Sfarčić, director of the Institute for the Study and Control of malaria in Trogir, in April 1924 brought from Spain several specimens of *G. holbrooki* and planted them in a small aquarium of Institute of Trogir where they thrived and increased *(12)*. After that mosquitofish were planted in a pond on island Čiovo, and in ponds of the area of Trogir. Antimalaric Institute of Trogir introduced *G. holbrooki* in 500 water recipients of the malaria infested area within the jurisdiction of the Institute in Trogir *(18)*. Later, in period 1940–1943, it was alaso widely introduced in Osijek and its surroundings (eastern Croatia) *(15)*. *G. holbrooki* originating from Virginia (USA) was transferred to Europe by Dr. Sella, then from Italy and Spain, to the Croatian regions of the Adriatic, and then to Bosnia and Herzegovina, Montenegro, Macedonia and Slovenia *(12)*. In an antimalaria campaign, *G. holbrooki* was introduced to 1973 locations *(18)*. In 1997 Peharda and Vrgoč *(19)* morphologically confirmed the presence of only *G. holbrooki* on island of Čiovo and in ponds in Hrvace (near Sinj). Both of these ponds were repeatedly treated with *Gambusia* by Sfarčić since 1925 *(15)*. Veenvliet 2007 *(20)* conducted a similar study in Slovenia and also morphologically confirmed to presence of only *G. holbrooki*.

European populations of *Gambusia* were long considered to be *G. affinis,* though the study of Vidal et al. 2010 *(5)* implied that they actually belong to *G. holbrooki*.

The traditional sister relationship of the *G. affinis* and *G. holbrooki* (*affinis* species-group) but also species monophyly were previously confirmed by phylogenetic analysis based on the mitochondrial cytochrome *b* gene *(21),* providing therefore the frame for genetic species identification. Based on the same molecular marker, the origin and genetic diversity of 24 European populations (from Portugal, Spain, France, Italy, Hungary and Greece) of *Gambusia* were recently investigated *(5).* This study revealed only the presence of four different haplotypes (Hol1, Hol3, Hol5 and Hol6) for *G. holbrooki* while *G. affinis* was not recorded.

The aims of this study were: 1) to contribute to knowledge on distribution of invasive *Gambusia sp.*, 2) the morphological and phylogenetic identification of the Croatian and Bosnian and Herzegovinian populations of *Gambusia* to determine whether they belong to *G. affinis* or *G. holbrooki* and 3) to obtain insight into phylogeographical structure of *Gambusia* in this region.

For this purpose, visual morphological determination was performed and the fragment of the mitochondrial cytochrome *b* gene was sequenced in individuals belonging to Croatian and Bosnian and Herzegovinian *Gambusia* populations and these were combined with previously published sequences of *G. affinis* and *G. holbrooki*.

MATERIALS AND METHODS

In this study, the literature data was analysed together with recent data collected from 2000 to 2014. The data was further analysed in ESRI ArcMap v. 10.2.2. software to plot aa distribution map of *Gambusia holbrooki* in Croatia and Bosnia and Herzegovina. Literature data was collected from following references: Plančić 1948 *(22);* Tutman 1948 *(23);* Sabioncello et al. 1964 *(24)*; Morović 1964 *(25)*; Habeković 1967*(26)*; Sabioncello 1967 *(27)*; Morović 1976 *(28)*; Bojčić et al. 1982 *(29)*; Leiner 1984 *(30)*; Treer et al. 1984 *(312)*; Leiner 1985 *(32)*; Tvrtković 1985 *(33)*; Treer 1989 *(34)*; Fašaić et al. 1990 *(35)*; Mrakovčić and Mišetić 1990 *(36)*; Leiner 1993 *(37)*; Vrgoč et al. 1994 *(38)*; Mrakovčić et al. 1995 *(39)*; Maitland and Crivelli 1996 *(40)*; Mrakovčić et al. 1996 *(41)*; Holčik and Mrakovčić 1997 *(42)*; Bonacci et al. 1998 *(43)*; Kerovec et al. 1998 *(44)*; Leiner 1998 *(45)*; Schneider 1998 *(46)*; Mrakovčić et al. 2000 *(47)*; Mikavica et al. 2001 *(48)*; Jakšić et al. 2008 *(9)*; Marguš 2008 *(49)*; Treer

TABLE 1 *Individuals molecularly analysed in the present study: geographical origins, sample codes, collection numbers (Tissue collection of the Croatian Natural History Museum), obtained cytochrome b (cytb) haplotypes and GenBank accession numbers. HR = Croatia, BiH = Bosnia and Herzegovina. Detailed geographic information is provided in Appendix 1 and Figure 1.*

Locality	Sample code	Collection number	*cytb* haplotype	GenBank Acc. No.
HR: Sinj, Otok, Karlauša – Sinjsko polje, channels	GKAR-1	CNHM TC11	A (HOL1)	KP723566
HR: Poreč, Valkarin pond	GKAR-3	CNHM TC1644	A (HOL1)	KP723565
HR: Bale	GKAR-4	CNHM TC1645	A (HOL1)	KP723564
HR: Pula, Štinijan	GKAR-5	CNHM TC1646	B (HOL5)	KP723559
HR: Rab, Fruga	GKAR-6	CNHM TC1647	A (HOL1)	KP723563
HR: Kukuletovica pond	GKAR-84	CNHM TC1810	A (HOL1)	KP723569
HR: Marceljani pond	GKAR-72	CNHM TC1798	A (HOL1)	KP723570
HR: Pekeci pond	GKAR-59	CNHM TC1785	A (HOL1)	KP723571
HR: Grego pond at Bale	GKAR-44	CNHM TC1770	A (HOL1)	KP723572
HR: Šalvela pond at Vodnjan	GKAR-26	CNHM TC1752	A (HOL1)	KP723573
HR: Galižana-Loborika	GKAR-99	CNHM TC1825	B (HOL5)	KP723560
HR: Krk, Vrbnik pond	GKAR-105	CNHM TC1831	A (HOL1)	KP723574
BIH: Neretva, Čapljina	GKAR-119	CNHM TC1929	A (HOL1)	KP723562
HR: ponds in Majkovi, Dubrovnik	GKAR-121	CNHM TC1931	A (HOL1)	KP723567
HR: Vrgorac, Matica	GKAR-122	CNHM TC1932	A (HOL1)	KP723568
HR: Čikola, Kljake	GKAR-106	CNHM TC1832	A (HOL1)	KP723556
BIH: Trebišnjica, Popovo Polje	GKAR-109	CNHM TC1835	A (HOL1)	KP723554
HR: Pag, salt lakes	GKAR-112	CNHM TC1838	B (HOL5)	KP723555
HR: Neretva, channels near estuary	GKAR-113	CNHM TC1839	A (HOL1)	KP723561
HR: Vransko Lake, channel Prosika	GKAR-117	CNHM TC1843	B (HOL5)	KP723557
HR: Mljet, lake in Blato	GKAR-115	CNHM TC1841	B (HOL5)	KP723558

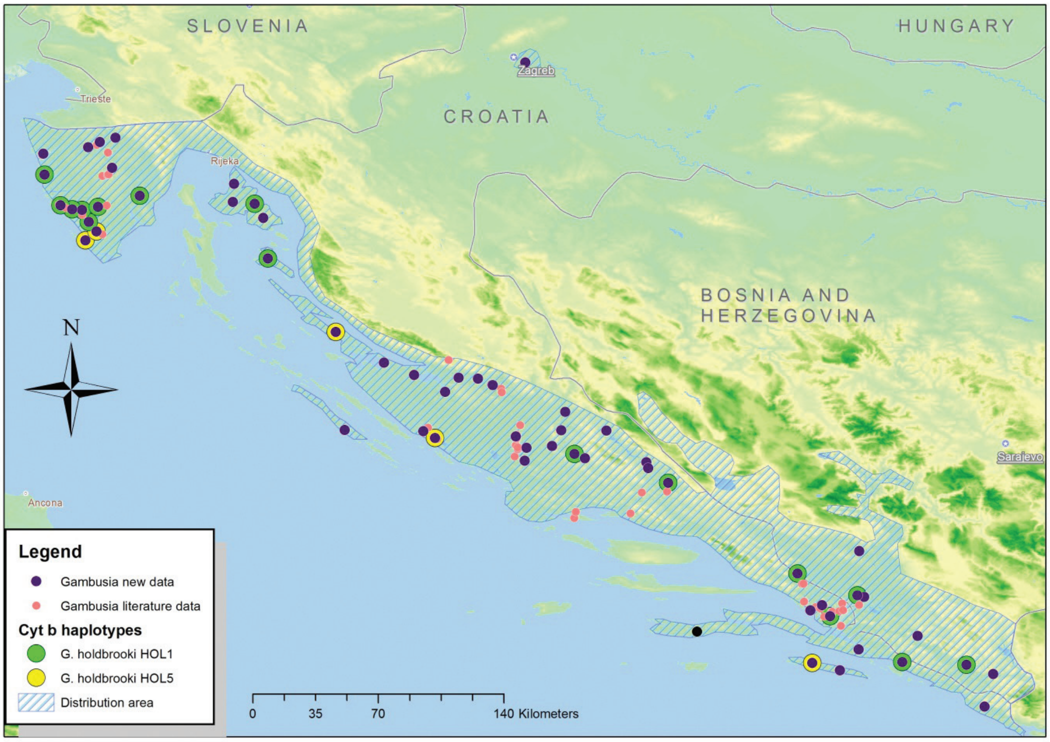

Figure 1. *Distribution of Gambusia holbrooki in Croatia and Bosnia and Herzegovina*

et al. 2010 *(50)*; Gabelica et al. 2011 *(51)*. A SAMUS 725M (max 1000 V, 600 W) electrofishing device and simple hand nets were used for sampling on altogether 63 locations (Appendix 1.). Tissue samples were taken from 21 location (Table 1.) and preserved fish specimens (male, and female) were taken from 10 populations (HR: Sinj, Otok, Karlauša – Sinjsko polje, channels; BIH: Neretva, Čapljina; HR: ponds in Majkovi, Dubrovnik; HR: Vrgorac, Matica; HR: Čikola, Kljake; BIH: Trebišnjica, Popovo Polje; HR: Pag, salt lakes; HR: Neretva, channels near estuary; HR: Vransko Lake, channel Prosika; HR: Mljet, lake in Blato).

For morphological inspection, the gonopodiums of preserved adult male *Gambusia* was inspected under a digital microscope (AM2111 Dino Lite Basic 20x-230x) and compared with the illustration by Veenvliet *(20)* and the description by Rosen and Bailey *(52)*. Morphological measurements were taken from 24 females and 23 males (representing all 10 localities) preserved in 70% ethanol during field research. This small subset of individuals was selected for inspection due to time consuming process of fixating and photographing the gonopodiums. All individuals were measured for weight, standard body length (SL) and total body length (TL). The number of rays in the dorsal (D) and anal (A) fins was counted according to Kottelat and Freyhof *(16)*. Morphological data collected here is only descriptive and is not intended to be used as general species morphological data.

Total genomic DNA was isolated from the muscle tissue of 21 samples collected in the course of this study from 21 geographic localities (Table 1, Figure 1) using PureLink Genomic DNA Mini Kit (Invitrogen). Although only the single individual per population was analysed, the sampling covered the great part of the *Gambusia's* range in the investigated area (Figure 1).

For all samples, the mitochondrial DNA fragment spanning from $tRNA_{Glu}$ to $tRNA_{Pro}$ and encompassing the entire cytochrome *b* (*cytb*) gene was amplified by polymerase chain reaction (PCR) using L14725 (5'-GAYTTGAARAACCAYCGTTG-3') and H15982 (5'-CCTAGCTTTGGGAGYTAGG-3') amplification primers *(53)*. The 50 µl PCR reaction mixture contained 1 x Go Taq®Reaction Buffer (containing 1.5 mM $MgCl_2$, Promega), 0.2 mM of each dNTP, 0.4 µM of each primer, 1.25 units of Go Taq®DNA Polymerase (Promega) and approximately 100 ng of genomic DNA. PCR cycling conditions comprised an initial denaturation step (94°C for 2 min) followed by 35 cycles of denaturation at 94° for 30 s, annealing at 50° for 30 s and elongation at 72° for 90 s and a final extension step of 72° for 7 min.

Sequencing was carried out by Macrogen Inc sequencing service (Seoul, South Korea) using L14725 as a sequencing primer (GenBank Accession Numbers: KP723554-KP723574). The obtained partial *cytb* sequences were edited and aligned using the BioEdit version 7.2.5 *(54)* and collapsed to haplotypes using FaBox *(55)*.

Tree-based species identification of the samples was performed by using three methods of phylogenetic inference: neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) as implemented in MEGA version 6 *(56)*. The complete *cytb* gene sequences of several *Gambusia* species were retrieved from the GenBank (GenBank Accession Numbers: KJ696803, EF017514, AP004422, JX679663, JX679662, EF017516, EF017515, EF017518) and aligned with haplotypes obtained in this study. The length of the final alignment was 1019 bp. The *cytb* sequences of *Belonesox belizanus*, *Girardinus rivasi* and *Poecila petenensis* (GenBank Accession Numbers: JX556410, FJ178707 and KJ696832, respectively) were used as outgroups *(57)*.

For MP analysis, the Tree-Bisection-Reconnection (TBR) algorithm and random addition of sequences (10 replicates) were used. ML analysis was based on the HKY+G model which was selected as the best-fit model of sequence evolution under the Bayesian information criterion. For all three methods, the clade support was evaluated by non-parametric bootstrapping (1000, 1000 and 100 pseudoreplicates for NJ, MP and ML, respectively). Additionally, a statistical parsimony network was constructed using TCS software v. 1.13 *(58)* under a 95% confidence limit. For this purpose *cytb* sequences from this study were aligned with all *G. holbrooki* sequences currently available in the GenBank (Accession Numbers: GHU18210, HM007038, HM443918 and HM443915 – HM443917, HQ609582 – HQ609589, JN565045, JN565047, JN565048 and KJ696803) and trimmed to 304 bp (the length of the shortest sequence). Haplotype frequencies were taken from Vidal et al. *(5)*.

This present study was performed with the authorization of Directorate for Nature Protection, Ministry of Culture (KLASA: UP/I-612-07/10-33/0345, URBROJ: 532-08-01-01/3-10-02; KLASA: UP/I-612-07l1 0-33/0616, URBROJ: 532-08-01-01/1-10-02; KLASA: UP/I-612-07/10-33/0719, URBROJ: 532-08-01-01/1-10-02).

RESULTS AND DISCUSSION

Distribution

Although *Gambusia* is a very common introduced species, there are surprisingly few literature records. Of the 149 records gathered for Croatia and Bosnia and Herzegovina, 58% (86) of were retrieved from the recent literature and 42% (63) were new records gathered by the authors. Distribution area presented in Figure 1 corresponds to the warmer areas close to the Adriatic shoreline. In this areas *G. holbrooki* finds sufficient amount of warm water habitats for reproduction. Only exception is one small population established in Savica Lake in Zagreb, which is a lake used for the discharge of hot water from the Zagreb heat plant. Otherwise they are not found in any of the other surrounding lakes. Gambusia was also widely introduced in Osijek and its surroundings (eastern Croatia) in period 1940–1943, but no literature or recent records of this species are available for anywhere in eastern Croatia. Three introduction events (Italy, 1924; Spain, 1924; USA, 1926) and main translocations known from the literature were summarized in Appendix 2. and compared with present distribution.

This species is still found in all localities in the Adriatic basin (Istria, Dalmatia, Herzegovina) mentioned in literature and also many more. It is obvious that *Gambusia holbrooki* has advanced capabilities of distribution into new water habitats, in many cases even microhabitats that are not used by other fish species (Mediterranean temporary ponds and swamps). The distribution and habitat preference of *Gambusia* are similar to those of several native and endemic fish such as *Gasterosteus gymnurus*, *Aphanius fasciatus*, *Telestes tursky*, *T. miloradi, Phoxinellus dalmaticus, P. alepidotus, Aulopyge huegelii*, *Scardinius dergle*, etc. To date there has been no systematic research conducted on how this aggressive invader impacts the endemic species communities. On two occasions, the authors accidentally housed *G. holbrooki* with endemic species in a 40 L tank for 7–12 days, and in both cases, they bit off pieces of caudal and anal fins until the death of the other species. In the first case, three adult females *Gambusia* killed three subadult *Phoxinus lumaireul* (2–4 cm total body length) and one adult (~6 cm). In the second case, one adult male and one female *G. holbrooki* killed two juvenile *A. huegeii* (2–3 cm) and one juvenile *S. dergle* (~4 cm). All juvenile individuals had the caudal fin completely bitten off, and the adult *P. lumaireul* also had pieces of the anal fin bitten off (2–3 mm) and it was apparent that the fish died of secondary infections. This kind of *Gambusia*-caused mortality has already been proven for endangered inanga fish (*Galaxias maculatus*) in New Zealand *(59)*. In nature, no injured or dead fish were observed, though in several small ponds (10 x 15 m) in the bed of the Čikola River near village Kljake (the river usually dries out in June or July), *T. tursky* and *P. dalmaticus* were found together with a hyperpopulation of *G. holbrooki* in July 2011. Later in August, no individuals of *T. tursky* or *P. dalmaticus* could be found at that site, just *G. holbrooki*. We have no knowledge of other developments that could happen between the two samplings and that would cause these species to disappear. This behaviour should be the subject of more detailed research due to the high number of stenoendemic fish species with a very small distribution are present in the Balkan Peninsula. There is a real danger that they could be pushed into extinction by an advanced invader like *Gambusia*.

Morphology of inspected individuals

All 23 inspected males from Croatia and Bosnia and Herzegovina had a gonopodium morphology which corresponds with *G. holbrooki*. In two individuals from

TABLE 2 *Morphological data on G. holbrooki Bosnia and Herzegovina individuals used in this study for gonopodium inspection and fin ray counts. SL = standard body length, TL = total body length. The length measurements are given in millimeters (mm) and weight measurements in grams (g).*

	Female			Male		
	N	Min – Max	Mean ± SD	N	Min – Max	Mean ± SD
SL	24	18.52 – 38.79	26.96 ± 5.852	23	17.41 – 26.76	21.81 ± 2.416
TL	24	22.86 – 47.93	33.41 ± 7.511	23	21.54 – 31.58	26.86 ± 2.717
Weight	24	0.11 – 1.20	0.43 ± 0.305	23	0.08 – 0.34	0.17 ± 0.067

Neretva River estuary the small spines on the third fin ray were not well visible, though this could be the consequence of preservation in a low ethanol dilution (material started to decompose). The hooks on the posterior branch of the fourth fin ray were relatively large, and compared to illustrations given by Veenvliet (2007) corresponded to *G. holbrooki* morphology. All inspected females had $6^{1/2}$ dorsal fin rays and $9^{1/2}$ anal fin rays, corresponding to *G. holbrooki*. All except four males had the same number of dorsal and anal fin rays ($6^{1/2}$ and $9^{1/2}$ respectively). One of these males (from Zrmanja River, Kaštel Žegarski) had $5^{1/2}$ dorsal fin rays that would correspond to *G. affinis*, but had $9^{1/2}$ anal fin rays. Three remaining males had broken dorsal fin so the number of rays could not been counted, but had $9^{1/2}$ anal fin rays.

All individuals selected for gonopodium inspection and fin ray counts (anal and dorsal) were adult specimens. Average standard length (SL) for males in our sample was 21.81 ± 2.416 mm and 26.96 ± 5.852 mm for females (Table 2). Smallest sexually mature individual was a male with SL of just 17.41 mm, and the largest individual was female with SL of 38.79 mm. Male average weight was 0.17 ± 0.067 g and for female this was 0.43 ± 0.305 g.

Genetic analysis

Over 1019 bp of the *cyt b* gene only two different haplotypes, A and B, differing by a single base substitution, were found (Table 1). In all phylogenetic trees they cluster with *G. holbrooki* sequence with high bootstrap support, proving, therefore, that all analysed samples belong to this species (Figure 2).

GenBank accession numbers are given in parentheses. Haplotypes obtained in this study (corresponding to Hol1 and Hol5 haplotypes of Vidal et al. *(5)* across the 309 bp long overlapping region) are depicted in bold. Numbers

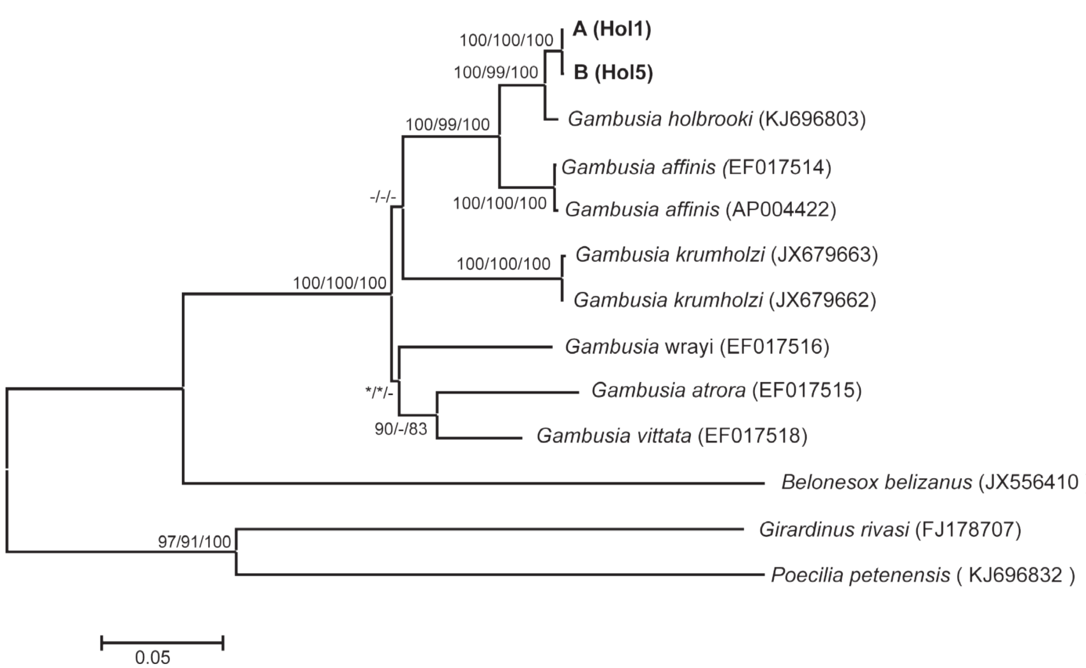

Figure 2. *Maximum likelihood phylogram based on 1019 bp long fragment of the mitochondrial cytochrome b showing the relationships between Gambusia specimens from this study and different Gambusia species.*

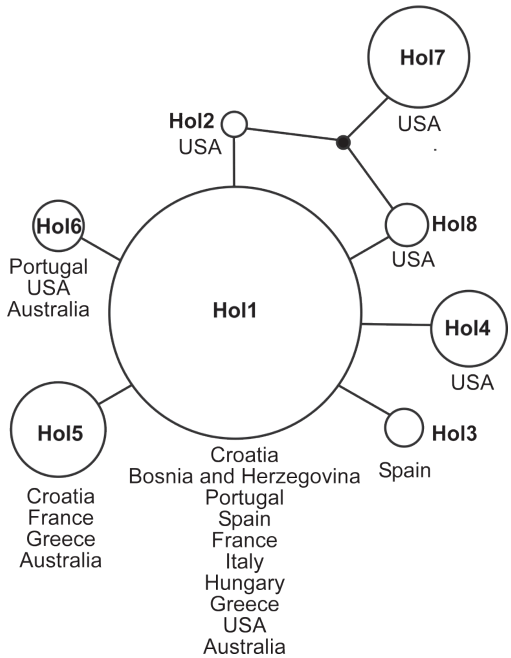

Figure 3. *Statistical parsimony network representing the relationships between Gambusia holbrooki cytochrome b haplotypes and their geographic distribution. The sizes of ovals reflect haplotype frequencies. Black dot represent missing intermediate.*

by the nodes represent bootstrap (BS) values (neighbor-joining/ maximum parsimony/maximum likelihood, respectively). BS values less than 70 are indicated by dash. * = node not obtained

Moreover, across the 309 bp long overlapping region, haplotypes A and B observed in this study correspond to Hol1 and Hol5 haplotypes *(5)*, respectively, which also represent the two most frequent and widespread haplotypes in European populations of *G. holbrooki* (Figure 3). While Hol1 haplotype was previously found in all investigated European populations with a single exception of one population from France, Hol5 haplotype was recorded for 2 French and 2 Greek populations.

In Croatia, those haplotypes (Hol1 and Hol5) do not show any regularity in their distribution. The haplotype Hol5 was found in Istria but also on Pag Island and as far away south as at Vransko Lake and Mljet Island. At all other localities haplotypes Hol1 was found.

Based on the observed distribution pattern they are most probably co-distributed within single populations as it is the case in France and Greece. However, since we analysed only the single individual per population in this study, it need to be confirmed by further investigations.

According to Vidal et al. *(5)*, the presence of Hol1 and Hol5 haplotypes in European populations of *G. holbrooki* can be ascribed to the two independent colonization events from the USA: Hol1 haplotype came in Europe in the course of the first introduction from the USA in Spain from where the fish were further transferred throughout Italy and many other European and Asian countries.

In the second introduction event, the Hol5 haplotype was introduced to France from where (as well as from Italy) the Greece was colonised with *G. holbrooki (5)*. The presence of both haplotypes (Hol1 and Hol5) in the Croatian region closely resembles to the pattern obtained in the Greece *(5)* and therefore strongly suggests the similar history of introduction.

Although *G. holbrooki* is very adaptable species it is obvious that it did not manage to make stabile populations in continental regions. In spite of intensive breeding and human dispersal, it did not manage to survive in Osijek area and establish natural population. In Zagreb it is only found in parts of Savica lakes where water temperature is increased during winter due to the permanent outflow of hot water from public heating system. This indicates that the cold continental climate in Croatia and Bosnia and Herzegovina is not favourable for overwintering or reproduction of *G. holbrooki*. Our results indicate that *G. affinis* introduced to Rovinj in 1926 *(15)* did not manage to establish its presence and was probably over dominated by already widespread *G. holbrooki*. This is why, later, it was not introduced to continental areas (Zagreb, Osijek) as *G. holbrooki* was. *G. affinis* is significantly more resistant to cold climate and could have been much more serious threat in continental Croatia and Bosnia and Herzegovina, but also central Europe. This species can still be found in pet trade (legal and illegal) and risk of introduction is still very high. All necessary measures should be taken into account to prevent this new invasion. *G. holbrooki* is now widespread in almost all natural and artificial water bodies in Mediterranean part of Croatia and Bosnia and Herzegovina. This species could be classified as one of the worst invasive species because of its variety of translocation vectors, speed of reproduction and direct predation on native fauna (also other fish).

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Appendix 1.

New data on distribution of *G. holbrooki* in Croatia and Bosnia and Herzegovina (N and E coordinates, in WGS84; presented as decimal degrees; UTM (10x10 km)). The data are presented in Figure 1.

Konavle, ponds and channels, 42,5507, 18,3253, BN81; Trebinje, 42,7133, 18,3679, BN83; Mljet, lake in Sobra, 42,7319, 17,6005, YH13; Trebišnjica, Popovo polje, 42,7591, 18,2346, BN73; Mljet, lake in Blato, 42,7680, 17,4618, YH03; ponds in Majkovi, Dubrovnik, 42,7735, 17,9120, YH33; Gorni Majkovi, two ponds, 42,7735, 17,9120, YH33; Ston, channels in village, 42,8368, 17,6942, YH24; Popovo polje, Trebišnjica, 42,9040, 17,9901, YH45; Korčula, Smokvica, 42,9350, 16,8820, YH06; Neretva, channels near estuary, 43,0030, 17,5511, XH96; Neretva estuary, 43,0322, 17,4519, YH07; Neretva, Modro oko, 43,0575, 17,5104, YH27; Bregava, Neretva, 43,0997, 17,7214, YH17; Neretva, Čapljina, 43,1077, 17,6874, XH98; Vrgorac, Matica, 43,2170, 17,3880, YJ10; Mostarsko blato, channel, 43,3292, 17,6966, XJ43; Sinj, Otok, Karlauša – Sinjsko polje, channels, 43,6716, 16,7388, XJ34; Rumin, channel near Cetina, 43,7753, 16,6299, XJ34; pond in Konjevrate, Krka, 43,7818, 16,0194, WJ84; Čikola, Čavoglave, 43,7945, 16,3216, XJ04; Čikola, Kljake, 43,8157, 16,2684, XJ05; pond in Ključ, Krka, 43,8465, 16,0290, WJ85; Čikola, Drniš, 43,8555, 16,1569, WJ95; Vransko jezero, channel Prosika, 43,8966, 15,5694, WJ46; Krka, Roški waterfall, 43,9034, 15,9753, WJ76; Vransko jezero, near ornithological station, 43,9301, 15,5117, WJ46; Vinalić, channels in the valley (Cetina), 43,9335, 16,4289, XJ16; River in Uzdolje (Knin), 43,9349, 16,2034, WJ96; Telašćica, pond near Sv. Ante church, 43,9366, 15,1175, WJ06; Krka, Šarena jezera in Knin, 44,0265, 16,2224, WJ97; Karin, Ravni Kotari, 44,1273, 15,6210, WJ48; Kaštel Žegarski, upstream from the bridge, 44,1608, 15,8596, WJ69; Zrmanja, Kaštel Žegarski, 44,1624, 15,8561, WJ69; Zrmanja, Berberi, 44,1931, 15,7859, WJ69; Zrmanja, Obrovac, 44,1988, 15,6879, WJ59; Brestica, Posedarje, 44,2114, 15,4648, WJ39; Jaruga, Miočići, Stošići, 44,2738, 15,3147, WK20; Pag, salt lakes, 44,4282, 15,0715, WK01; Rab, Fruga,44,0331, 14,7463, VK76; Pula, Štinijan, 44,8873, 13,8164, VK07; Galižana-Loborika, 44,9315, 13,8729, VK17; Šalvela pond at Vodnjan, 44,9798, 13,8332, VK08; Krk, Ričina, Baška, 44,9996, 14,7091, VK78; Bale, 45,0398, 13,8005, VK08; Grego pond at Bale, 45,0431, 13,7511, VK08; Pekeci pond, 45,0541, 13,8797, VK18; Kukuletovica pond, 45,0622, 13,6917, UK99; Krk, Vrbnik pond, 45,0702, 14,6655, VK79; Krk, lake Ponikve at Malinska, 45,0788, 14,5567, VK69; Marceljani pond, 45,1099, 14,0898, VK29; Jezero at Njivice, 45,1698, 14,5620, VL60; Poreč, Valkarin pond,45,2057, 13,6446, UL90; Pazinčica, 45,2507, 13,9505, VL11; Mirna, estuary, 45,3210, 13,6050, UL91; Mirna, Stara Mirna at Livade, 45,3536, 13,8301, VL02; Istarske Toplice, Mirna, 45,3796, 13,8895, VL12; Mirna, Buzet, 45,4014, 13,9680, VL12; Savica, Zagreb, 45,7793, 16,0226, WL77.

Appendix 2.

Map of three main introduction routes for *Gambusia sp.* and main known translocations routes from 1924 until 1943 within research area.