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‘Trojan fish’: Exploring haplotypic and genotypic diversity of introduced *Gambusia holbrooki* in India

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Abstract Assessing haplotypic and genotypic diversity is crucial for comprehending biological invasion dynamics and devising effective management strategies. In India, *Gambusia* spp. were introduced in the early twentieth century for mosquito biocontrol, but recent studies have demonstrated their detrimental impact on native biota. The present research employs molecular tools to investigate the haplotypic and genotypic diversity of *Gambusia* species in India. The findings reveal a greater distribution of *Gambusia holbrooki* and a lesser occurrence of *Gambusia affinis* in India. The study underscores that *G. holbrooki* was initially brought into India from Europe and later multiple times from other regions across the globe. Our global haplotypic network shows the presence of a single *G. affinis* haplotype, which is narrowly distributed in the northeastern part of India. Additionally, our population genetic analyses exhibit a low genetic variability and significant population genetic structuring among *G. holbrooki* populations. This study emphasizes the significance of evaluating haplotypic and genotypic diversity to develop a comprehensive understanding of the invasion history and dynamics of introduced species, thereby aiding in the development of effective management strategies that mitigate their negative impact on native biota.

Keywords *Gambusia holbrooki* · *Gambusia affinis* · Haplotypic diversity · Multiple introductions · Genetic diversity

Introduction

Invasive alien species (IAS) have been known to negatively influence ecosystem functioning and species richness in invaded locations (Vilà et al., 2011; Mollot et al., 2017). IAS are defined as species that have been introduced beyond their natural range, can sustain a fertile population over several generations in their introduced range, can significantly expand their range from the site of introduction, and have the potential to cause negative impacts on local ecosystems, human health, and the economy (Hui & Richardson, 2017). In recent decades, the rapid escalation

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of human activity worldwide has led to a surge in the transportation of numerous species, resulting in both intentional and unintentional introduction of IAS across the globe (Duenas et al., 2021). Upon successful establishment, these alien species may undergo range expansion, thereby invading local habitats and potentially outcompeting native species (Nobinraja & Ravikanth, 2020).

IAS represent the second biggest threat to ecosystems worldwide and have been reported to cause extensive economic damage (Pejchar & Mooney, 2009). In Europe, IAS resulted in a total economic loss estimated at US$140.20 billion (or Euro 116.61 billion) from 1960 to 2020 (Haubrock et al., 2021). In Asia, a total loss of US$432.6 billion was reported between 1965 and 2017, with India alone losing around 176.7 US$ billion (Liu et al., 2021) due to IAS. Similarly, economic losses in the UK were estimated to be $6.9 billion to $17.6 billion (£5.4 billion–£13.7 billion) between 1976 and 2019 (Cuthbert et al., 2021). Globally, the estimated loss due to aquatic IAS was US$345 billion during the period 1971 to 2020 (Cuthbert et al., 2021). Freshwater ecosystems face significant threats, including biological invasion, yet remain understudied (Reid et al., 2019; Duenas et al., 2021; Roy et al., 2021). Fish introductions are widespread globally, driven by aquaculture and the ornamental trade (Gozlan et al., 2010). India ranks third in economic costs associated with Invasive Alien Fishes (IAF) (Cuthbert et al., 2021). Understanding invasion dynamics, influenced by introduction pathways, genetic diversity, and interactions with biotic and abiotic factors is crucial for effective management and biodiversity conservation (Tsutsui et al., 2003; Arim et al., 2006; Hulme, 2009; Lora & Robert, 2013). Moreover, it is an important tool to comprehend the evolutionary process and success of the biological invasion (Suarez & Tsutsui, 2008).

Among the IAF in India, the western mosquitofish *Gambusia affinis* (Baird & Girard, 1853) and the eastern mosquito fish *Gambusia holbrooki* Girard, 1859, are small freshwater fishes belonging to the family *Poeciliidae*. These two species were introduced in freshwater ecosystems around the world for the biocontrol of mosquitoes in malaria control programs due to their presumed ability to control mosquito larvae (Saç, 2023; Gao et al., 2019). Their broad diet, physiological tolerance, rapid population growth, high genetic variability, aggressive behavior, and dispersal tendencies make the mosquitofishes highly adaptable to different environments (Pyke & White, 2000; Pyke, 2008; Stockwell & Henkanaththegedara, 2011; Singh et al., 2013). *Gambusia* spp. were reportedly introduced into India in the early twentieth century for the biocontrol of malarial mosquitoes (Nobinraja & Ravikanth, 2020). *G. affinis* and *G. holbrooki* are native to Eastern parts of North America. The natural distribution range of *G. holbrooki* is east and of *G. affinis* is west of the Appalachian Mountains. Both species are widely recognized as IAS due to their extensive distribution beyond their native range (Nobinraja & Ravikanth, 2020). The introduction of *G. affinis* and *G. holbrooki* into various ecosystems has resulted in their widespread proliferation, leading to significant negative impacts on native biodiversity, and ecosystems, and incurring substantial economic costs (Hurlbert & Mulla, 1981; Nagdali & Gupta, 2002; Macdonald & Tonkin, 2008; Moore et al., 2008; Pyke, 2008; Stockwell & Henkanaththegedara, 2011; Špoljar et al., 2019; Brinsden, 2020). The invasion of *G. affinis* and *G. holbrooki* has inflicted ecological damage, including the decline of native fish species due to competition for resources, predation on fish and amphibian eggs, larvae, and adults, the transmission of parasites and diseases, as well as aggressive behavior resulting in injury and mortality to a number of faunal taxa, and hybridization (Stockwell & Henkanaththegedara, 2011). Furthermore, *Gambusia* has demonstrated negative impacts on invertebrates, plants, and amphibians (Pyke, 2008; Stockwell & Henkanaththegedara, 2011).

However, the accurate identification of these species is a major concern, as females are cryptic and difficult to distinguish from males without the presence of a gonopodium (Holland et al., 2004; Cabrera et al., 2017). *G. affinis* and *G. holbrooki* have also caused taxonomic ambiguity due to their similar morphology and utilization as mosquito control agents (Lee, 2014), presenting obstacles in managing and controlling these invasive alien species. Furthermore, *Gambusia* spp. have been extensively utilized in various scientific studies, including toxicology, behavior, physiology, and microbiome research, both in India and other countries (Reddy & Kote, 1975; Rehage & Sih, 2004; Begum et al., 2006; Leonard et al., 2014; Nobinraja & Ravikanth, 2020; Liu et al., 2022). Given the wide range of applications and implications of these studies, misidentifying *Gambusia* spp. can lead to erroneous data interpretation and potentially hinder the progress of scientific investigations.

There have been cases of misidentification of *Gambusia* spp. in different regions of the world. In Australia, previous records mistakenly identified Australian specimens as *G. affinis* until the 1980s, when they were correctly reclassified as *G. holbrooki* (Cabrera et al., 2017). Similarly, in Europe, historical documents indicated the introduction of both species in 1920, but subsequent genetic analyses conducted in six European countries exclusively confirmed the presence of *G. holbrooki*. In Argentina, specimens were consistently designated *as G. affinis*; however, further examination revealed that they belonged to *G. holbrooki*. In India, *G. affinis* is the predominantly reported *Gambusia* spp. according to reports but recent evidence has indicated a wide presence of *G. holbrooki* in India (Nobinraja & Ravikanth, 2020). The invasion history of *Gambusia* spp. in India is also poorly documented. The major narrative suggests that *G. affinis* was introduced from Italy to Bangalore, in 1928 (Nobinraja & Ravikanth, 2020). It is assumed that it has likely spread from Bangalore to other parts of the country. However, this has been contested by various authors (Krishnaja & Rege, 1983; Singh & Gupta, 2010, 2014). Ensuring accurate species identification is of paramount importance for effectively managing cryptic species, implementing targeted control measures, and detecting introductions. In this regard, the molecular approach has emerged as one of the most promising and robust methods for achieving accurate information (Ayres et al., 2010, 2012; Vidal et al., 2010; Cabrera et al., 2017).

By employing molecular approaches, researchers can not only ensure precise species identification but also gain critical insights into population dynamics through the analysis of haplotypic and genotypic diversity. This knowledge plays a pivotal role in providing valuable insights into various aspects of their population dynamics including bottleneck effects, and genetic clustering. Genetic diversity assessment is vital for understanding population health, adaptability, and survival of *Gambusia* spp. in new environments (Prentis et al., 2008). Genetic analysis, including comparing genetic structures (Biedrzycka et al., 2014), bottleneck analysis (Guillemaud et al., 2010), and clustering (Boissin et al., 2012), helps determine introduction history, population dynamics, and connectivity, facilitating effective management and conservation strategies (Howell et al., 2013; Mandal, 2011). In the current study, we focus on investigating the haplotypic and genotypic diversity of *Gambusia* spp. in India and explore the distribution as well as the invasion route. In particular, we seek to address: (a) which species of *Gambusia* were introduced to India; (b) is there evidence of multiple introductions of *Gambusia* in India, and what is their haplotypic diversity; (c) do any of the introduced populations display signs of a recent bottleneck effect; and finally (d) what are the levels of genetic diversity across the introduced populations of mosquitofish in India?

Materials and methods

Sample collection and DNA extraction

A total of 300 individual mosquitofish specimens were collected across 16 different sites spanning nine states and one union territory in India (Table 1; Fig. 1). Sampling locations were narrowed down using extensive scientific and grey literature surveys. Sample collections were conducted between December 2019 and December 2021 after obtaining the necessary permissions. Sampling was carried out between 6:00 AM and 2:00 PM due to the diurnal nature of the fish. Fishes were collected using a handheld dip net (mesh size ~ 1 mm) from various water bodies such as lakes, private ponds, and drainages at different elevations (Table 1). Collected mosquitofish were anesthetized in 0.2% Tricaine, euthanized onsite, and preserved in > 95% ethanol after incubation in an ice slurry. They were later stored at − 20 °C for molecular analysis. Morphological inspection of the gonopodium of preserved males was conducted under a light microscope (Fig. 2). The genomic DNA was extracted from ethanol-preserved fish tissue samples using the QIAGEN kit (blood and tissue) (Catalogue number 69504). The final elution was performed twice with 50 µL elution buffer and stored at − 20 °C until further processing. Isolated genomic DNA was amplified with selected mitochondrial and microsatellite markers (Table 2).

Mitochondrial DNA and sequencing analysis

A total of 97 mosquitofish specimens from 16 populations were chosen for mitochondrial DNA analysis.

Table 1 Sample collection locality (with type, latitude and longitude and elevation), haplotypic variation in Cytb

Locality (no. of samples)	State/Union territory (total no. of samples)	Species	Haplotype name (no. of individuals)	Elevation (m)	Waterbody type	Latitude	Longitude
Lalbagh, Bangalore (13)	Karnataka (26)	*Gambusia holbrooki*	Hol 5, Hol 1, Hol6	912	L, PP, PP, L	12.9479264	77.5815305
Dharwad (8)				745		15.4390894	74.98478
Nagarhole (4)				864		11.992521	76.0648098
Malavali, Mandya (1)				621		12.3942569	77.0463875
Chennai (8)	Tamil Nadu (18)	*Gambusia holbrooki*	Hol 5, Hol 1, Hol6	4	D, L, L	12.9216384	80.2324823
Ooty (5)				1995		11.4664122	76.5626758
Madurai (5)				142		9.977886	78.14906
Hyderabad (5)	Telangana (5)	*Gambusia holbrooki*	Hol 1	557	L	17.4340036	78.3911884
Vizagapatnam (5)	Andhra Pradesh (5)	*Gambusia holbrooki*	Hol 1, Hol6	20	L	17.7926391	83.1898013
Karyavattom (5)	Kerala (5)	*Gambusia holbrooki*	Hol 1, Hol6	48	PP	8.565329	76.8868848
Mumbai (5)	Maharashtra (10)	*Gambusia holbrooki*	Hol 5, Hol 1	2	PP	19.1797220	72.9901737
Pune (5)				564		18.529156	73.842401
Kolkata (10)	West Bengal (10)	*Gambusia affinis*	Aff1	0	L	22.530664	88.460057
Tura (8)	Meghalaya (8)	*Gambusia holbrooki*	Hol 5	408	L	25.5709429	90.2389253
Phek, Kami (5)	Nagaland (5)	*Gambusia affinis*	Aff1	1702	D	25.5292972	94.2662305
Delhi (5)	Delhi (5)	*Gambusia holbrooki*	Hol 1	230	L	28.589246	77.1902996

The table shows the sample collection locality (with type, latitude and longitude and elevation), haplotypic variation in Cytb for *Gambusia holbrooki* and *Gambusia affinis* collected from different states/union territories in India from 2019 to 2022. The waterbody types are denoted as *L* Lake, *PP* Private pond, *D* Drainage. The haplotype names are abbreviated as Hol followed by a number, and Aff followed by a number for *G. holbrooki* and *G. affinis*, respectively. The data indicates the presence of three haplotypes of *G. holbrooki* and one haplotype of *G. affinis* in India

Isolated DNA was subjected to mtDNA cytb gene amplification, as suggested by Vidal et al. (2010). PCR amplification was carried out with 11 µl of 2 × Amplicon Master-mix, 0.5 µl of 10 pmol CytBF1, 0.5 µl of 10 pmol CytBR1, 0.5 µl of template DNA and a total reaction volume of 25 µl was made up with ddH$_2$O. PCR cycling conditions were: an initial three min denaturation step at 94 °C for one cycle, followed by 35 cycles of 94 °C for 30 s, 51.2 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min. The amplification of the PCR products was verified on a 2% agarose gel. Final amplified fragments were sequenced with cytb primers. The cytb amplicons generated were ~ 370 bp, but were trimmed to restrict the length of the amplicon to 304 bp for comparison with global data. The cytb sequences were manually edited and aligned using Chromas 2.6.6 (Technelysium Pty Ltd., Tewantin, Australia). and Mega7 (Kumar et al., 2016). Reference cytb sequences of *G. affinis* and *G. holbrooki* were obtained from the NCBI database (Online resource Table S1). The cytb sequences were compared with 13 *G. affinis* sequences from the USA and Taiwan, as well as 37 sequences of *G. holbrooki* from Europe, the USA, and Australia available from GenBank (Online resource Table S1) to verify species identity. Phylogenetic analysis was carried out using maximum likelihood (ML) using a General Time Reversible model (GTR) with gamma-distributed rates and with 1000 bootstrap replicates in RaxML v.2.4.4 (Guindon et al.,

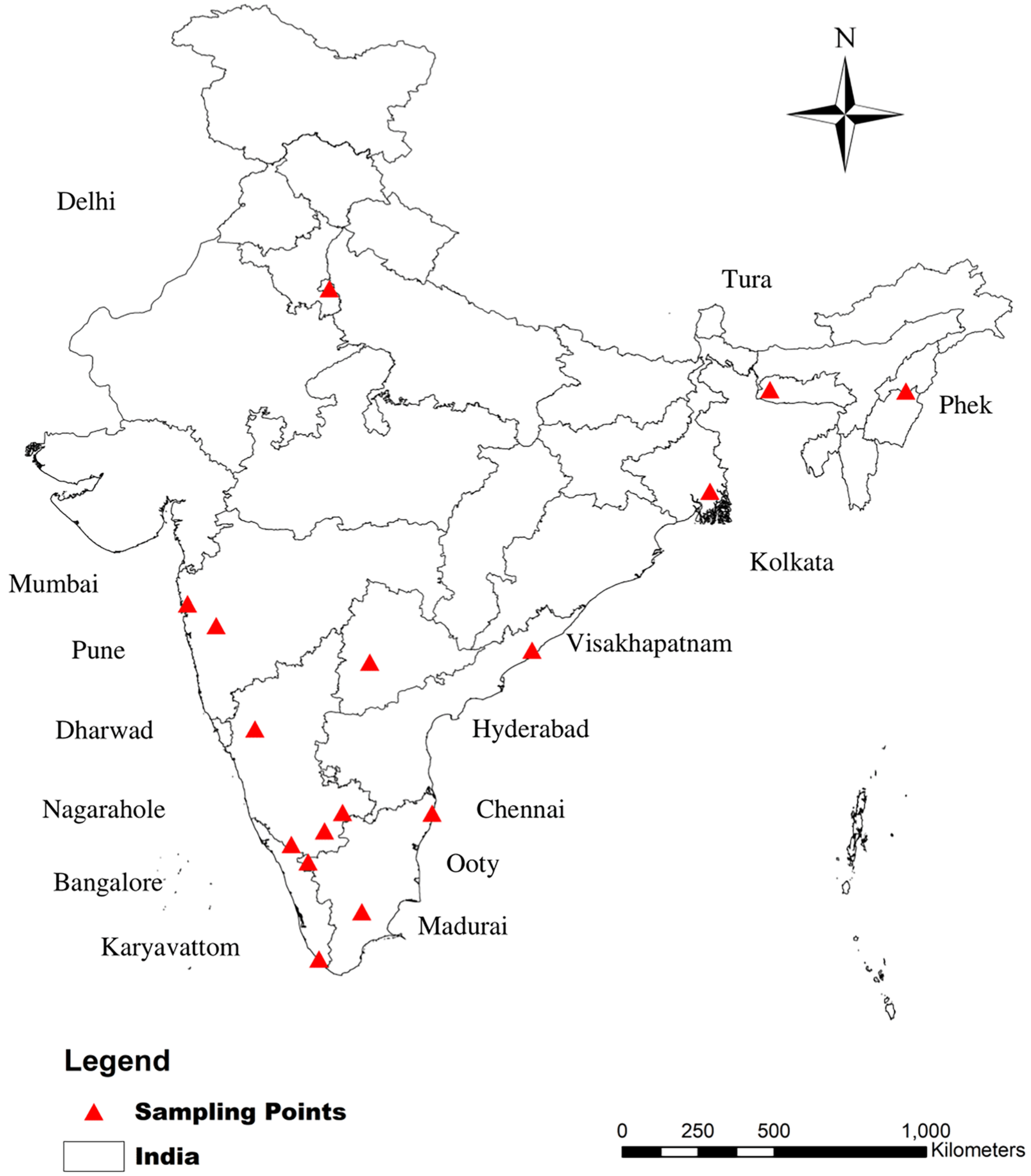

Fig. 1 Sample collection location of *Gambusia* spp. The map shows the sampling locations for *Gambusia* spp. in India between 2019 and 2022. The locations include Bangalore, Dharwad, Nagarhole, and Mandya from Karnataka; Chennai, Ooty, and Madurai from Tamil Nadu; Hyderabad from Telangana; Vishakapatnam from Andhra Pradesh; Karyavattom from Kerala; Pune and Mumbai from Maharashtra; Kolkata from West Bengal; Tura from Meghalaya; and Kami from Nagaland, and Delhi. The map was generated to provide a visual representation of the distribution of *Gambusia* spp. in India

2005). Haplotype diversity and frequencies were calculated using DNAsp V5 (Librado & Rozas, 2009) and Arlequin 3.5.2.2 (Excoffier & Lischer, 2010) (Online resource Table S2). A global cytb haplotype network based on a median-joining network was created using Popart 1.7 (Leigh & Bryant, 2015).

Microsatellite analysis

A total of 220 individuals, with 20 samples from each of the 11 different populations, were screened using eight microsatellite loci markers developed for *G. holbrooki* (Umbers et al., 2012) (Table 3). The PCR amplifications were performed in a 10 µl total reaction volume containing: 1 µl of template DNA, 3.5 µl of Type-it® master mix, 1 µl of end-labeled forward primer, 1 µl of unlabeled reverse primer and the volume made up to 10 µl with ddH_2O. The microsatellite loci were amplified following PCR cycling conditions: Initial denaturation at 95 °C for 5 min, followed by 28 cycles of 95 °C for 30 s, 60 °C for 90 s, 72 °C for 30 s, with the final extension at 60 °C for 30 min. The amplified samples were subjected to genotyping on Applied Biosystems 3730xl DNA analyzer. As a result of the limited sample size and relatively low distribution of *G. affinis* in India, the species was excluded from the microsatellite analysis. The haplotypic network analysis also revealed a lack of significant genetic variation among *G. affinis* populations, which further supported the decision to exclude the species from the microsatellite analysis. The focus of the study was on addressing the major issues related to *G. holbrooki* and its genetic diversity, given the current distribution and its spread scenario. While the decision to exclude *G. affinis* may limit the study's ability to provide a comprehensive understanding of the *Gambusia* invasion in India, the study aimed to provide detailed insights into the genetic diversity of *G. holbrooki* in the region.

Statistical analysis

The genotyped samples were manually evaluated and scored using Genemarker V2.6.3 (Hulce et al., 2011). Numbers of alleles, the effective number of alleles, private alleles, Shannon's Information Index, Fixation Index, Nei's genetic distance, and observed and expected heterozygosity were calculated using GenAlEx (6.5) (Peakall & Smouse, 2012) (Online resource Table S3). Genetic differentiation, genotypic disequilibrium and F statistics were calculated and tested using FSTAT, Version 2.9.3 software (Goudet, 2001). Pairwise F_{st} and hierarchical AMOVA were calculated using ARLEQUIN v3.5 (Excoffier & Lischer, 2010). The genetic relatedness of the eleven populations was assessed using Principal Component Analysis (PCA) using Genodive (3.0) (Meirmans, 2020) and Origin pro b Version 2019b (OriginLab Corporation, Northampton, MA, USA). Geographic clustering between populations was analyzed using the Bayesian clustering approach STRUCTURE v.2.3.3 (Pritchard et al., 2000). The populations were evaluated under an admixture and non-admixture model. The algorithm estimates allele frequencies for each gene pool (cluster) and population memberships for every individual. The analyses were performed with 200,000 and 100,0000 MCMCM repeats after burn-in with ten iterations for each K value (1–15). The number of clusters was assessed using Structure Harvester to estimate the ΔK value according to Evanno et al. (2005). Hardy–Weinberg Equilibrium was analyzed using PopGene (Version 1.32) (Yeh et al., 1999). The occurrence of any recent bottleneck effect was tested using BOTTLENECK Version 1.2.02 (Cristescu et al., 2010).

Results

Sequencing results

The Maximum Likelihood Tree built using 170 cytb sequences (97 generated in this study) among all the *Gambusia* species shows the presence of only two *Gambusia* spp. in India (Online resource Fig. S1). Samples collected from Kolkata, West Bengal and Nagaland clustered together with *G. affinis* sequences (Fig. 3). Meanwhile, mosquitofish populations from Karnataka, Tamil Nadu, Andhra Pradesh, Telangana, Delhi, Meghalaya, Kerala, and Maharashtra cluster together with *G. holbrooki* sequences. *G. affinis* and *G. holbrooki* are sister species and form a monophyletic clade. For the haplotypic network analysis, only 152 sequences (of *G.affinis* and *G. holbrooki*) were used. Between these two species, a total of 14 different haplotypes of the *cytb* gene were identified from 152 sequences obtained from Europe, India, Australia, the USA and Taiwan (Online resource Table S1). Of these 152 sequences, 97 were from India (this study), 21 were from the USA, 25 were from various parts of Europe, four were from Australia and one was from Taiwan. The global haplotypic network showed gaps in data availability worldwide for mtDNA cytb diversity of introduced *G. affinis* and *G. holbrooki* (Fig. 2). Only five different haplotypes of *G. holbrooki* have been reported from the USA and these include Hol1 (South Carolina, Mississippi, North Carolina, Washington D.C), Hol2 (North Carolina), Hol4 (South Carolina), Hol6 (Washington D.C), Hol7 (Florida), Hol8 (Florida). In Europe, Hol1 (Greece, Croatia, Rhodes, Portugal, Spain, France, Italy, Hungary, Bosnia and Herzegovina); Hol3 (Spain); Hol5 (Croatia, France, Greece) and Hol6 (Portugal) have been reported. Australia has three haplotypes Hol1 (HM007038), Hol5 (JN565047) and Hol6 (JN565048) (Vidal et al., 2010).

In the case of *G. affinis*, six haplotypes namely: Aff1 (Mexico, Mississippi, New Jersey, Oklahoma); Aff2 (San Francisco, Texas, Oklahoma); Aff3 (Texas); Aff4 (Mississippi); Aff5 (Texas); and Aff6 (Texas) have been reported from the USA. From Taiwan, a single haplotype (Aff1) has been reported (Chang et al., 2019).

From India, four haplotypic variations in the 304 bp mtDNA Cytb region were observed. The haplotypes were Hol1, Hol5, and Hol6 of *G. holbrooki* and Aff1 of *G. affinis* as mentioned in Vidal et al. (2010). Hol1 is the most found haplotype throughout the world and in India and comprises 45.1 percent. Forty-five out of 97 sequences represent Hol5 and Hol6 in India; 24 sequences represent Hol5 (24.7 percent) and 21 sequences (21.6 percent) were of Hol6. A total of 15 individuals collected from Nagaland and West Bengal confirmed the presence of *G. affinis* with a single Aff1 haplotype showing no haplotypic diversity. Hol2-6 and 8 are separated from Hol1 by a single mutation. This indicates that these haplotypes could have originated from Hol1; Hol1 being widespread indicates that it may be the oldest haplotype. The mutations observed between Hol1 and Hol5 are differentiated due to the GA transition at the 282nd position. Similarly, Hol1 and Hol6 differ due to a TC transition at the 41st position in the cytb gene (Fig. 2).

Microsatellite results

In the genotype data, a total of 35 alleles were observed for eight microsatellite loci in 11 populations (220 individuals) of *G. holbrooki*. The number of unique alleles ranged from 1.50 in Hyderabad to 3.875 in Bangalore. The average number of alleles in the entire population was 2.557 (Table 3). Between the populations, Hyderabad and Tura had the least number of effective alleles; meanwhile, Bangalore and Chennai had the highest with a mean across all the loci of 1.853 ± 0.078. Our data showed that observed and expected heterozygosity ranged between 0.038–0.326 and 0.123–0.584 across populations. The number of alleles was highest in Bangalore, while observed heterozygosity was highest in Karyavattom, Kerala. Private alleles were detected in populations in Bangalore and Mumbai. Estimates of observed heterozygosity across all the loci and populations (0.214 ± 0.02) indicate a low genetic diversity among the populations. The signature of a recent bottleneck event was detected under IAM in Chennai, Karyavattom and Delhi (Table 3).

Genetic differentiation and population structure

On average, the percentage of polymorphic loci across populations was 81.82 indicating that a high proportion of loci showed genetic variation. The average number of alleles per locus is 4.375 ± 0.5 (Online resource Table S4 and S5). The F_{is} or inbreeding coefficient values were 0.43 ± 0.03 indicating a moderate level of inbreeding in the populations (Table 3 and online resource Table S5). The F_{is} was lowest in Hyderabad and Ooty (0.204 ± 0.1 and 0.255 ± 0.1) while the highest inbreeding was observed in Madurai (0.664 SE 0.1) and Madurai also shows a low level of observed heterozygosity (0.151 ± 0.06).

The STRUCTURE analysis on the 11 populations found the strongest support to differentiate clusters ($K = 2$) in both admixed and non-admixed populations. The first cluster comprised populations from Tura, Bangalore, Chennai, Karyavattom, Madurai, Ooty, and Visakhapatnam populations while Nagarhole (outskirts), Hyderabad, Delhi and Mumbai clustered into another (Fig. 4). The PCA analysis revealed similar clustering among the populations of *G. holbrooki* in India (Online resource Fig. S2). The results of global Analysis of Molecular Variance (AMOVA) indicate that 58 percent of the genetic variations in *G. holbrooki* are contained within populations ($F_{\mathrm{is}} = 0.351$; $F\mathrm{sc} = 0.366$; $F\mathrm{ct} = 0.085$ and F_{it}: 0.624; $P < 0.001$), while the proportion of genetic variance partition among populations was 42 percent ($P < 0.001$, Table 4 and Online resource Table S4). This suggests that there is a significant genetic differentiation between the populations of *G. holbrooki*, and this could be due to factors such as geographic isolation or limited gene flow between populations.

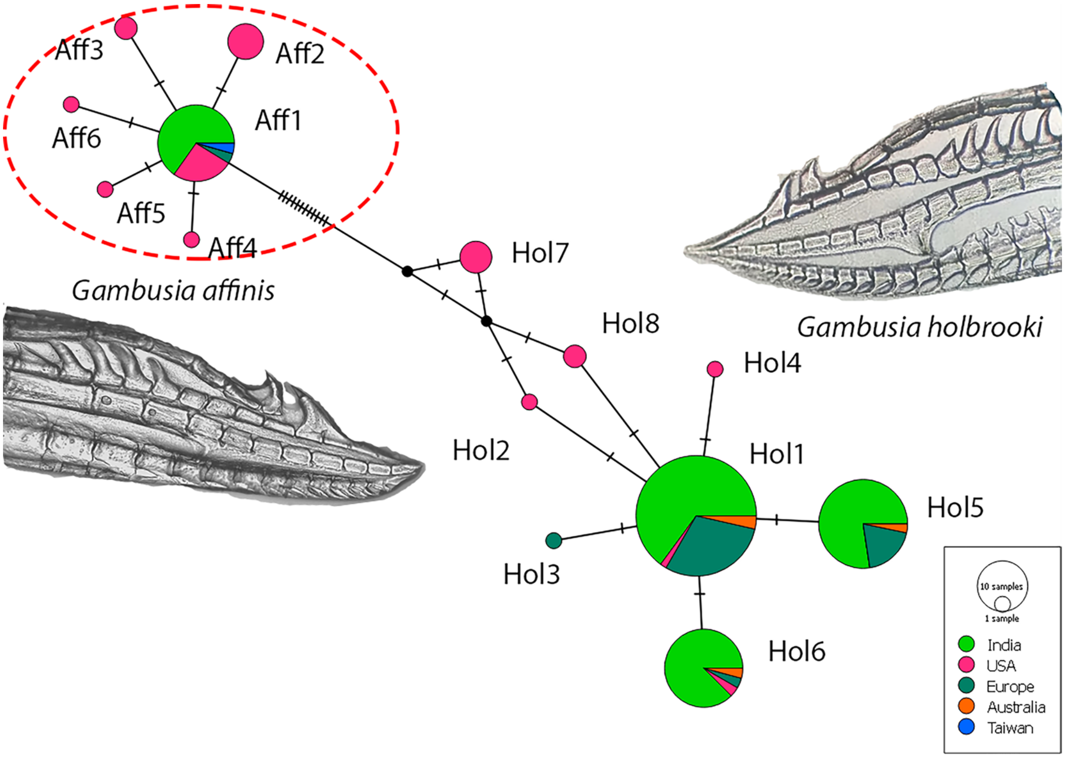

Fig. 2 Global network containing haplotypes of *Gambusia holbrooki* and *Gambusia affinis* and microscopic images of the gonopodium of *G. affinis* and *G. holbrooki*. This figure shows a global haplotypic network containing haplotypes of *Gambusia holbrooki* and *Gambusia affinis*, along with a microscopic image of the gonopodium of *Gambusia affinis* on the left and *Gambusia holbrooki* on the right. The network displays eight haplotypes of *G. holbrooki* and six haplotypes of *G. affinis* from various locations around the world. The size of each circle represents the number of times a particular haplotype was reported. This information aids in understanding the global distribution and genetic diversity of these two species of *Gambusia*

Results for each of the 8 loci across all the populations are presented in the online resource, Table S5. The global deficit of heterozygotes across populations (F_{IT}) ranged from 0.414 to 0.778. An overall significant ($P < 0.05$) deficit of heterozygotes (F_{IS}) of 0.438 (ranging from 0.138 to 0.665) was observed in the analyzed loci. The deficit of heterozygotes also indicates low genetic diversity within the populations.

Discussion

G. affinis and *G. holbrooki* are two of many globally introduced IAF (Srean, 2015). Mosquitofish have been reported as one of the top 100 worst IAS on earth due to their aggressive behavior in the introduced localities (Lowe et al., 2000). Studies across the world have shown the detrimental impact of their voracious feeding behavior and their impact on endemic and endangered species (Pyke, 2005, 2008; Moore et al., 2008). Thus far, it has been unclear as to which species of *Gambusia* was introduced into India (Nobinraja & Ravikanth, 2020). From the available literature, it was also presumed that a single introduction was carried out which resulted in *Gambusia* spreading across India (Krishnaja & Rege, 1983; Singh & Gupta, 2014, 2016). Despite the designation of *Gambusia* spp. as an IAF by the National Biodiversity Authority (NBA), various central and state government bodies continue to introduce the species for mosquito control (Nobinraja & Ravikanth, 2020).

Our study was aimed at resolving the taxonomic ambiguities about the species of *Gambusia* widely found in India and to understand the introduction pathways of *Gambusia* spp. Our results indicate that *G. holbrooki* was first introduced into India from

Table 2 Primer sequences used for PCR and microsatellite analysis

Locus	Repeat motif	Allele size range
Locus 01	(AGAT)12	129–195
Locus 14	(ATG)11	118–136
Locus 19	(AAT)12	245–311
Locus 22	(TAT)25	155–194
Locus 27	(AC)22	140–150
Locus 35	(TGT)18	81–129
Locus 37	(GAT)18	89–149
Locus 41	(ATT)13	90–120
mt-gene	**Primer**	**Sequence (5′ to 3′)**
Cytb	CytBF1	(5′-ATGGCC AAC CTA CGA AAA AC-3′)
	CytBR1	(5′- GGG TAG RAC ATA ACC TAC GAA G-3′)

Primer sequences used for microsatellite analysis and mtDNA−Sequencing. The microsatellite primers were selected from Umbers et al. (2012) and Mt gene primers were used from Vidal et al. (2010). The table provides information on the repeat motif and allele size range for each locus, as well as the primer sequences (5′–3′) used for the mt−gene fragment amplification

Europe and not *G. affinis,* as was previously reported (Ghosh et al., 2012). Studies from Europe indicate the presence of only *G. holbrooki* and according to the earlier literature, *Gambusia* spp. were introduced from Italy to Lalbagh Lake in the 1920s (Pyke, 2008; Vidal et al., 2010; Sanz et al., 2013; Nobinraja & Ravikanth, 2020; Pirroni et al., 2021). Our analysis from Lalbagh also indicates the presence of only *G. holbrooki,* not *G. affinis*. Our pan-India sampling showed the presence of *G. holbrooki* (87.5%) as a dominant species and *G. affinis* is only found in two populations. Several earlier surveys and research articles published throughout India report the presence of *G. affinis,* which we presume is largely due to misidentification (Nobinraja & Ravikanth, 2020).

Our results show the presence of three out of eight haplotypes reported for *G. holbrooki* in India and only one of the six haplotypes for *G. affinis*. Recent literature suggests the subsequent introduction of *Gambusia* spp. to the Kashmir valley from Italy and again in 1968 from Italy and Thailand (Aislabie et al., 2019). In 1968, two introductions in Hyderabad and Andhra Pradesh were reported, but their origin is unknown (Aislabie et al., 2019). This literature evidence fails to explain the wide distribution of *G. holbrooki* and its multiple haplotypes. From our analysis, it is clear that Italy only has haplotype Hol1, which is the dominant haplotype found in India, but apart from that, there are two more (Hol5 and Hol6) haplotypes widespread across India. Europe, in total, reported the presence of four haplotypes of *G. holbrooki* and none of *G. affinis* (Vidal et al., 2010). Haplotypes Hol1 and Hol5 are quite common throughout Europe; however, haplotype Hol6 was sampled only in Portugal. Meanwhile, the USA, which is part of the native range of *G. holbrooki,* is reported to have six haplotypes (Vidal et al., 2010). This denotes the complex introduction routes of *G. holbrooki* to India.

The southern Indian city of Chennai, located in the state of Tamil Nadu, is shown to harbor all three haplotypes of the invasive *G. holbrooki*. Notably, Kolathur in Chennai is home to one of the largest ornamental fish trades in Tamil Nadu, consisting of 150 fish-producing units that commonly breed a variety of ornamental fish species, including the mosquitofish (Shinoj et al., 2021). Due to their low cost (approximately 0.012 USD per fish), *Gambusia* spp. are often used as feeder fish for larger aquarium species such as Arowana (*Osteoglossum* spp.). It is plausible that Chennai served as one of the gateways for multiple introductions of *G. holbrooki* into India.

In Chennai, Hol1 and Hol6 occur at 37.5 percent frequency and Hol5 at 25 percent. Bangalore (Lalbagh), supposedly, where the species was first introduced, harbors 61.5 percent of Hol5 and 38.5 percent of Hol1. Ooty and Visakhapatnam share two of the same haplotypes but Hol6 is dominant in Ooty and Hol1 is dominant in Visakhapatnam. Tamil Nadu, Andhra Pradesh and Kerala are the major

Table 3 Genetic variation in microsatellite data summary statistics

Population		N	Na	Ne	I	Ho	He	uHe	F	P	IAM	SMM
Tura	Mean	19.750	1.625	1.198	0.216	0.038	0.131	0.134	0.557	0.5	0.281	0.305
	SE	0.164	0.263	0.097	0.098	0.025	0.061	0.063	0.167			
Bangalore	Mean	18.875	3.875	2.632	1.050	0.325	0.584	0.600	0.416	1.0	0.071	0.095
	SE	0.549	0.227	0.241	0.107	0.066	0.059	0.061	0.101			
Chennai	Mean	17.750	3.125	2.383	0.923	0.321	0.539	0.555	0.437	1.0	0.044*	0.079
	SE	1.264	0.295	0.267	0.107	0.069	0.054	0.054	0.100			
Karyavattom	Mean	19.875	2.375	2.172	0.780	0.326	0.513	0.526	0.378	1.0	0.002*	0.004*
	SE	0.125	0.263	0.217	0.091	0.081	0.040	0.041	0.130			
Nagarhole	Mean	18.750	1.875	1.364	0.350	0.113	0.217	0.224	0.564	0.62	0.414	0.145
	SE	0.726	0.295	0.136	0.112	0.060	0.072	0.074	0.147			
Madurai	Mean	15.250	3.125	1.964	0.746	0.151	0.414	0.429	0.664	0.87	0.299	0.310
	SE	1.859	0.398	0.251	0.156	0.065	0.089	0.092	0.112			
Ooty	Mean	17.250	2.750	1.756	0.612	0.285	0.362	0.372	0.255	0.87	0.522	0.369
	SE	1.278	0.366	0.220	0.135	0.104	0.082	0.084	0.148			
Visakhapatnam	Mean	18.125	2.750	1.953	0.724	0.303	0.444	0.456	0.317	0.87	0.074	0.643
	SE	1.355	0.366	0.195	0.121	0.081	0.069	0.070	0.131			
Hyderabad	Mean	19.500	1.500	1.196	0.199	0.102	0.123	0.126	0.204	0.50	0.405	0.339
	SE	0.327	0.189	0.117	0.088	0.062	0.061	0.062	0.188			
Delhi	Mean	18.875	2.750	2.054	0.760	0.203	0.464	0.477	0.619	1.0	0.004*	0.251
	SE	0.718	0.250	0.280	0.104	0.066	0.056	0.057	0.122			
Mumbai	Mean	16.875	2.375	1.708	0.552	0.191	0.327	0.336	0.381	0.75	0.097	0.553
	SE	0.875	0.460	0.268	0.155	0.057	0.087	0.089	0.128			
Average	Mean	18.261	2.557	1.853	0.628	0.214	0.374	0.385	0.439	81.82		
	SE	0.315	0.115	0.078	0.044	0.022	0025	0.026	0.039	5.93		

This table presents the genetic variation summary statistics for different populations studied in the research. Total of 220 individuals, with 20 samples from each of the 11 different populations, were screened using eight microsatellite loci markers developed for *G. holbrooki.*The populations studied are Tura, Bangalore, Chennai, Karyavattom, Nagarhole, Madurai, Ooty, Visakhapatnam, Hyderabad, Delhi, and Mumbai. The table provides information on the number of individuals (N), number of alleles (Na), effective number of alleles (Ne), Shanon diversity index (I), observed heterozygosity (Ho), expected heterozygosity (He), unbiased expected heterozygosity (uHe), fixation index (F), and percentage of polymorphic loci (P). The table also includes the results of the tests for the Infinite Allele Model (IAM) and Stepwise Mutation Model (SMM) ($P > 0.05$). The mean and standard error (SE) for each parameter are presented for each population. The average values for each parameter across all populations are also provided

centers for the aquaculture industry and hence could have had multiple introductions resulting in more than one haplotype in these regions. Hol1 is present in lower elevations of Mumbai, Chennai, Karyavattom, Visakhapatnam and Hyderabad extending to Dharwad, Bangalore, and even in the higher elevations of Ooty. Hol5 is distributed in Tura, Bangalore, Pune and Chennai. Meanwhile, Hol6 is in Nagarhole, Ooty, Chennai, Madurai and Visakhapatnam.

Studies from Asia (Taiwan, Philippines, Japan, China, Thailand, and Bangladesh) have shown the presence of *G. affinis* and not *G. holbrooki* (Lee, 2014; Hossain et al., 2019). *G. affinis* found in Kolkata and Nagaland could have been introduced either from Southeast Asia or from regions of Mexico, Mississippi, New Jersey, or Oklahoma, which make up its native range. There are established ornamental and aquaculture trading networks between countries like China and Bangladesh, which could have been the major introduction pathway for *G. affinis* into India. *G. affinis* is largely distributed in China and it was first introduced in that country in 1924 from Taiwan and again in 1927 from the Philippines (Lee, 2014). It is important to note that India is one of the very few countries in the world where both *G. affinis* and *G. holbrooki* have been reported.

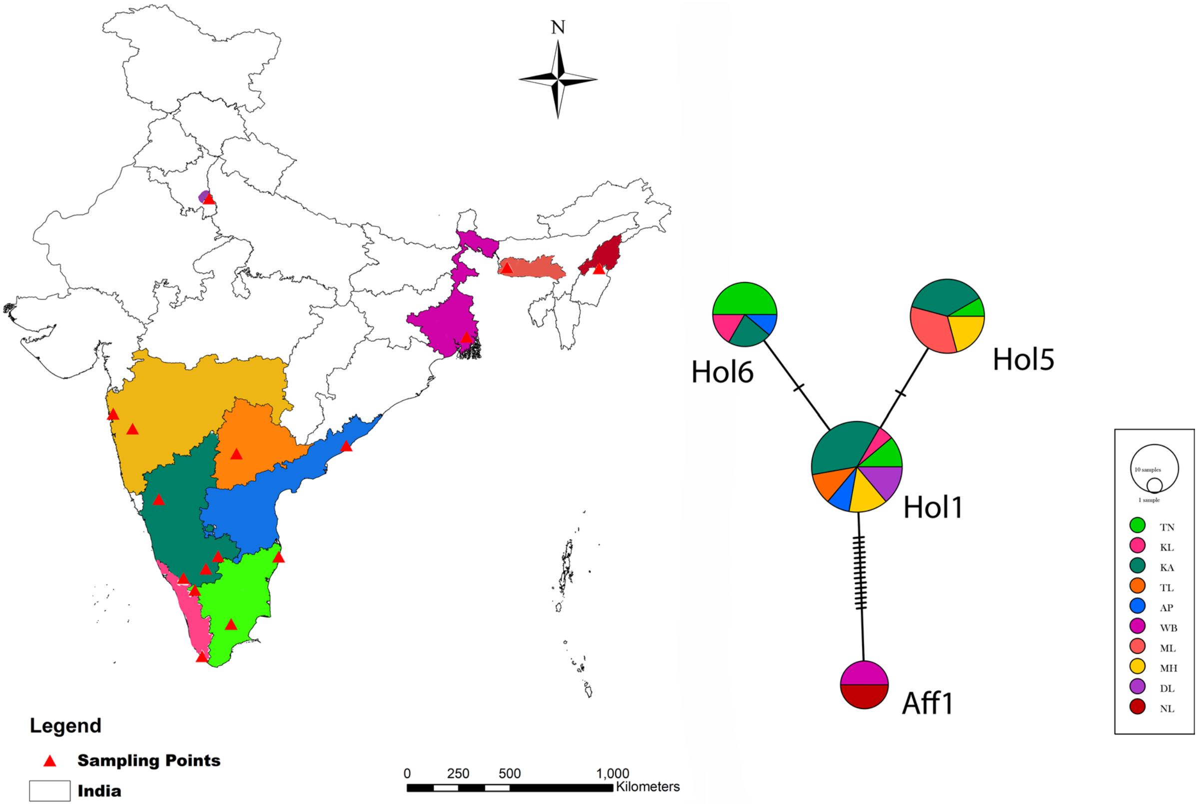

Fig. 3 Median-joining network of *Gambusia holbrooki* and *Gambusia affinis* haplotypes in India. This figure presents a median-joining network illustrating the distribution of haplotypes of *Gambusia holbrooki* and *Gambusia affinis* in India. The different colors on the network represent various states in India, including Tamil Nadu (TN), Kerala (KL), Karnataka (KA), Telangana (TL), Andhra Pradesh (AP), West Bengal (WB), Meghalaya (ML), Maharashtra (MH), Delhi (DL), and Nagaland (NL). The network displays the presence of three haplotypes of *G. holbrooki* and one haplotype of *G. affinis* in India. Interestingly, *G. affinis* was only found in the northeast regions of the country, while *G. holbrooki* was widespread across India. Additionally, the size of each circle in the network is directly proportional to the number of individuals containing that haplotype

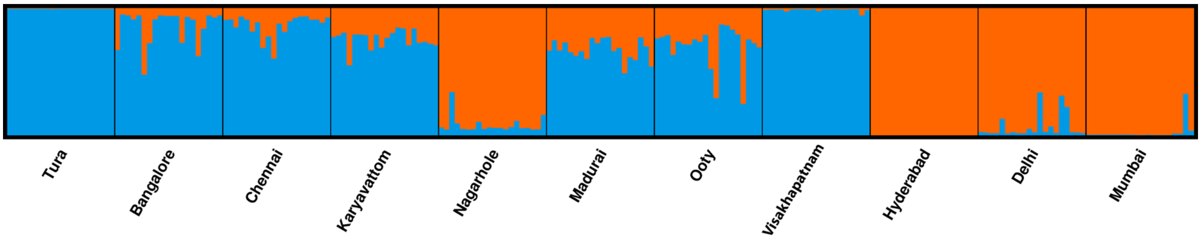

Fig. 4 Population clusters of *Gambusia holbrooki* identified using microsatellites ($K=2$). This figure depicts the population structure of *Gambusia holbrooki* using microsatellite analysis, where $K=2$ was used to identify two distinct population clusters depicted in blue and orange. The identified populations are Tura, Bangalore, Chennai, Karyavattom, Nagarhole, Madurai, Ooty, Vishakapatnam, Hyderabad, Delhi, and Mumbai. The first population cluster comprised Tura, Bangalore, Chennai, Karyavattom, Madurai, Ooty, and Visakhapatnam populations. On the other hand, Nagarhole, Hyderabad, Delhi, and Mumbai populations clustered into another group. This information is vital in understanding the genetic structure and diversity of *G. holbrooki* populations in India

Table 4 Genetic variation in allele data from *Gambusia holbrooki* using analysis of molecular variance (AMOVA)

AMOVA design and results

Source of variation	Sum of squares	Variance components	Percentage variation
Among groups	34.3	0.09302	8.60
Among populations within groups	138.0	0.36218	33.47
Among individuals within populations	177.0	0.22004	20.33
Within individuals	89.5	0.40682	37.60
Total	438.9	1.08206	

This table shows the results of the analysis of molecular variance (AMOVA) for genetic variation in allele data from *Gambusia holbrooki*. The table presents the sources of variation, sum of squares, variance components, and percentage variation for each source. The table shows the sum of squares and variance components for each source of variation, as well as the percentage variation that each source contributes to the total variation. The total sum of squares and variance components are also presented. The results indicate that there is a significant amount of genetic variation within populations and also some genetic differentiation between populations

In India, the occurrence of hybrids between *G. affinis* and *G. holbrooki* remains a possibility, albeit with limited evidence (Wilk & Horth, 2016). While our study suggests *G. holbrooki* as the predominant species introduced in India, of European origin despite being native to the USA, some parts of the country may likely harbor hybrids due to multiple introductions from different source populations. For instance, areas with overlapping populations of both *G. affinis* and *G. holbrooki* might provide opportunities for hybridization (Wilk & Horth, 2016).

To better assess the chances of hybridization between *G. affinis* and *G. holbrooki* in India, a comprehensive taxonomical examination, including gonopodium examination, fin ray assessment, and genetic-based confirmation using appropriate markers, such as microsatellites, should be carried out (Scribner & Avise, 1994; Vidal et al., 2010; Ayers et al., 2011; Wilk & Horth, 2016). This approach will aid in accurately identifying and differentiating between the two species, providing insights into the potential for hybridization and the genetic composition of *Gambusia* populations. Furthermore, conducting mate choice studies and compatibility assessments will be valuable in exploring the possibility of hybridization and its implications for the distribution and ecological dynamics of *Gambusia* spp. (Morgado-Santos et al., 2015; Stelkens et al., 2015) in the country. Through robust taxonomical and genetic analyses, a deeper understanding of the hybridization potential and its relevance to local aquatic ecosystems could be achieved (Wilk & Horth, 2016).

Our study also revealed low genetic variation, indicating reduced genetic diversity within populations, and overall loci F_{st} was significantly different from zero ($F_{st}=0.3$) (Table 3). Observed heterozygosity values ranged from 0.038 to 0.326, indicating moderate inbreeding in the populations. The mean heterozygosity (Ho = 0.21) of the introduced *G. holbrooki* was significantly lower than the average reported in Australia (Ayres et al., 2012). The observed high F_{st} in *G. holbrooki* populations in India may be attributed to the possibility of multiple introductions of the species into the region, suggesting a more complex invasion history than previously thought (Peery et al., 2012; Hagenblad et al., 2015; Du et al., 2016). Our analysis found little evidence of a genetic bottleneck, but the Chennai, Karyavattom, and Delhi populations showed recent bottleneck effects under the IAM model, and Karyavattom under the SMM model (Table 3). Despite the genetic bottleneck that introduced species often undergo, they eventually adapt to new environmental factors and become successful invaders due to various phenomena (Jeschke, 2014).

The STRUCTURE analysis revealed two major clusters ($K=2$) that could not be explained solely based on geographical location. However, we propose that Chennai and Bangalore are major contributors to the populations in neighboring states extending up the east coast, while Mumbai may be the major contributor for the second cluster, as these cities are major routes for the ornamental fish trade (Satam et al., 2018).

Most of the southern and eastern regions of India form one cluster (Ooty, Madurai, Visakhapatnam, Chennai, Bangalore, Karyavattom and Tura) while the northern and western populations (Delhi, Nagarhole, Mumbai and Hyderabad) form another cluster. This may be attributed to the aquarium trade and multiple stakeholders introducing these fishes as part of malaria control programs (Ghosh et al., 2012).

The present study investigated the genetic diversity and population structure of *G. holbrooki*, an invasive alien fish species in Indian watersheds. The results indicated low genetic diversity and higher genetic structuring in the Indian populations of *G. holbrooki*, which is consistent with the findings of previous studies conducted on this species in other regions of the world. The low genetic diversity observed in the Indian populations of *G. holbrooki*, compared to the native populations, may be attributed to founder events, genetic bottlenecks, and limited gene flow. These factors are known to play significant roles in shaping the genetic diversity of invasive populations. The study findings are in agreement with Ayres et al. (2010), who reported low genetic variation and higher genetic structuring of *G. holbrooki* in the Greater Melbourne area, Australia, where the fish was introduced from Italy around the same time as in India. Similarly, Lindholm et al. (2005) reported the invasion success of *P. reticulata* in Australia despite low genetic diversity. The low genetic diversity in invasive populations has also been reported in other studies, including Tsutsui et al. (2000), Koskinen et al. (2002), Roman & Darling (2007), Purcell et al. (2012), & Hagenblad et al. (2015). The present study is consistent with the findings of Vera et al. (2016), who reported that genetic drift was the primary evolutionary force affecting the genetic diversity and population structure of invasive *G. holbrooki* populations introduced from North America to Europe. Additionally, the present study findings are consistent with those of Grapputo (2006), who reported a strong reduction in genetic diversity and heterozygosity in four European populations of *G. holbrooki* using the RAPD PCR method.

Our findings are consistent with Ayres et al. (2012) study, which also found low genetic diversity and strong genetic patterns in *G. holbrooki* populations in Australia. The study also revealed evidence of recent bottleneck events, which may explain the reduced genetic diversity in the population. However, it is worth noting that the use of mtDNA and microsatellite markers presents certain limitations when compared to SNPs, as reported by Vidal et al. (2011). In contrast, their study on *G. holbrooki* populations in Europe found higher levels of genetic variation using SNP markers.

In conclusion, the results of our study demonstrate that genetic drift and founder events may have influenced the genetic diversity and population differentiation of invasive *G. holbrooki* populations in India. Multiple introductions have likely contributed to the complex invasion history of the species, and the remarkable invasion success of *G. holbrooki* in India may be due to species traits, such as its rapid breeding and high dispersal ability, as well as human-mediated translocation. During our sample collection, the fishes were spotted across a wide range of elevations, indicating their ability to adapt and spread. However, despite the observed genetic constraints, ongoing monitoring and management strategies are necessary to minimize the negative impacts of introduced species on native ecosystems (Díez-del-Molino et al., 2013). Additionally, although the haplotypes observed globally match those present in India, the history of the introduction of *G. holbrooki* to India remains unclear. The use of mtDNA and microsatellite markers in our study has limitations compared to SNP markers, which have been shown to provide higher levels of genetic variation in European populations of *G. holbrooki* (Vidal et al., 2011). Further genetic data using SNP markers, which are recently developed for *G. holbrooki*, may be useful to identify and understand genetic patterns associated with the introduction and spread of this species in more detail. Such information will help understand the processes facilitating the successful invasion of *G. holbrooki* worldwide and inform management actions.

Importance of preventing further introductions

The negative impacts of mosquitofish have been studied at multiple sites. An experimental study conducted by Vannini et al. (2018) reported the potential impact of *Gambusia* spp. on native European amphibians. Studies have shown that when *G. holbrooki* interacts with native fishes, it displays highly aggressive behavior such as fin nipping. It is also reported that habitat degradation and severe decline in surface flow in waterbodies will benefit *G. holbrooki* to the detriment of native fishes (Beatty et al., 2022). The extensive negative impacts observed in multiple sites and the aggressive behavior displayed by *Gambusia* spp. towards native species justify labeling it as a “Trojan fish”, reminiscent of the mythical strategy where the entry of seemingly harmless objects led to invasive consequences. In this case, the introduction of *Gambusia* as a mosquito control agent resulted in its establishment and subsequent invasive behavior in waterbodies, posing threats to native biodiversity and ecosystems.

An introduced population of alien species goes through a bottleneck that can reduce the fitness and evolutionary potential of the species (Estoup et al., 2016). However, IAS often overcome this due to multiple introductions and multiple source populations (Díez-del-Molino et al., 2016). This creates genetic variability in the introduced population in a short period of time, equipping the IAS to adapt to various conditions. Genetic admixture between multiple source populations can also give rise to a wider tolerance range for the IAS (Estoup & Guillemaud, 2010). *G. holbrooki* is a successful invasive alien fish species in India and this could be due to human-mediated multiple introductions from diverse regions (Díez-del-Molino et al., 2016). The viviparous mosquitofish has a wider tolerance range for several abiotic factors (Kurtul et al., 2022). It is reported from temperatures ranging between 0 and 45 °C, highlighting its versatility (Rollings et al., 2014). Studies have shown some individuals of mosquitofish can survive even below 0.2 °C (Wood et al., 2020). The species has been reported to survive a 0–41 ppt salinity level, pH ranging from 4.5 to 9.0, and they can survive dissolved oxygen levels ranging from 1 to 11 mg/l (Pyke, 2008; Gao et al., 2017). Recent studies have shown sustained aerobic performances with hypoxia tolerances well suited for biological invasion success (Callaghan et al., 2021), making them a successful agent in biocontrol and one of the most widespread invasive alien fishes across the globe, except Antarctica (Pyke, 2008). It has been hypothesized that mosquitofish adapt well in isolation and when presented with extreme environments (Pyke, 2008; Wood et al., 2020). According to a study conducted in Iran, the increasing impact of climate change can potentially have significant consequences on the survival of *Gambusia* and *Bufotes* species (Taheri Khas et al., 2019), underscoring the need for further investigations to assess the potential for future invasions. Furthermore, Santi et al. (2020) demonstrated that *G. holbrooki* can adapt to novel environments through a combination of genetic changes and phenotypic plasticity, highlighting the importance of both mechanisms in facilitating successful range expansions. These findings contribute to our understanding of the potential impact of environmental changes on IAS, and the importance of considering both genetic and plastic factors in predicting their future spread.

In conclusion, our study shows evidence for multiple introductions of *G. holbrooki* in India from multiple source populations. While the time of introduction events is not very clear, our findings suggest that *G. holbrooki* has quite successfully established across India. However, despite multiple introductions, the nuclear genetic diversity for *Gambusia* spp. are low. This suggests the possibilities of phenotypic plasticity and standing genetic variation leading to the successful invasion of *G. holbrooki* in India.

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Author contributions MN and GR contributed to the study conception and design. Material preparation, data collection and analysis were performed by MN. The first draft of the manuscript was written by MN and GR commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The gene sequence data obtained and generated during the current study are available in the National Center for Biotechnology Information—NCBI (https://www.ncbi.nlm.nih.gov/) and the accession numbers are in the manuscript and supplementary information.

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

Research involving human participants and/or animals The authors followed all institutional and/or national guidelines for sample collection during the course of this study. The Institutional Animal Ethics Committee has reviewed and approved the sample collection methods (No: AAEC/107/2018).

Informed consent Not applicable.

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