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Research Article

Molecular Identification of Several Morphologically Distinct Flowerhorn Fish (Family) Using Mitochondrial *COI* Gene Marker

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ABSTRACT
Flowerhorn fish has been known as a breed of fish produced by artificial hybridization between several cichlid fish. Other ornamental cichlid fish generally known to be crossed to make flowerhorn variant includes *Amphilophus citrinelus*, *Amphilophus labiatus*, *Vieja melanurus*, and *Amphilophus trimaculatus*. Our study identified a variety of flowerhorn samples with distinct morphotypes, dubbed as Cencu (LH1CC), Kamfa (LH2KF), Thai Silk (LH3TS), Kirin (LH4KR), Parrot (LH5PR), and Vieja (LH6VJ) using mtDNA *COI*-based DNA barcoding. Molecular analysis and phylogenetics showed that all sample had 0% genetic divergence and conspecific with *A. trimaculatus* sequence. Hence, we concluded that despite having varied morphotypes, all flowerhorn samples were identified as *A. trimaculatus* and were a variation of flowerhorn from *A. trimaculatus* lineage. The findings should be used as a precaution as the fish is identified as an invasive species.

INTRODUCTION
Ornamental fish has been one of the most prominent live fish commodities in the ornamental market. The global market valuation for ornamental fish is estimated at more than US$ 10 billion, with over 2500 species being traded and more than 60% are freshwater fish (Dey et al. 2016). Among the freshwater fish breed and marketed as ornamental fish, flowerhorn fish is popular as aquarium fish especially in Southeast Asia (Mutia et al. 2007; Nico et al. 2007; Ng 2016).

Flowerhorn fish is considered as a hybrid individual, being a cross between different species and breeds of Neotropical cichlids that originated in Central America, such as Parrot cichlid, *Amphilopus labiatus*, *A. citrinelus*, *Amphilophus trimaculatus*, and *Vieja melanurus* (Nico et al. 2007; McMahan 2010). It is a popular aquarium fish known to be bred with various other cichlid species to produce individuals with specific desirable characteristics. The fish were divided into numerous types in the market based on morphological traits such as body color, pattern, and the presence of a head hump. The flowerhorn varieties were dubbed with various names such as Kamfa, Nakeemix, Golden base, Zhen zhu (Cencu) (Gonzales-Plasus et al. 2022).

Flowerhorn fish is a medium-sized fish with adult size around 35 cm (Magalhães et al. 2017), had vivid coloration and is often characterized by its head protuberance or nuptial hump and has black horizontal markings on their body (Than et al. 2019). Flowerhorn fish have been traded and distributed throughout Asia, the Americas, and Europe as decorative aquarium fish. The fish trade was particularly high in the Southeast Asia (Sandford 2007), with many aquarium owners and traders specially breed and contesting their fish. The flowerhorn itself is not a fish naturally found in local environment. In light of this, releasing the fish into nearby rivers, lakes, and waterways could potentially introduce an invasive species that endangers the aquatic habitat there (Mutia et al. 2007).

The invasive characteristics of flowerhorn fish derived from its hardiness and high-adaptability, making it a prominent competitor to the local fish community. The fish has been known as highly aggressive, omnivorous, adaptable and can rapidly breed in natural environment due to its parental brood care trait (Knight 2010). It is also predatory especially to juveniles and smaller fish, hence not only putting ecological pressure through resource competition but also through predation of the endemic fish (Hilgers et al. 2018). Cases of flowerhorn presence in local environment have been documented in the Southeast Asia region, such as in Malaysia (Ahmad et al. 2020), Thailand (Nico et al. 2007), and the Philippines (Guerrero 2014).

The flowerhorn fish is also widely circulated and traded in Indonesia as ornamental fish, marketed as "Louhan fish", and is also considered a non-native and invasive species (Sentosa & Hedianto 2019). There have been several studies documenting the invasion of flowerhorn fish in local water system and natural lakes in Indonesia. The presence of flowerhorn varieties has been reported in Cirata reservoir in West Java (Wahyuni et al. 2014), Sermo Reservoir in Yogyakarta (Ariasari et al. 2018), Lake Toba in North Sumatra (Sinambela et al. 2021), and Lake Maninjau in West Sumatera (Samir et al. 2021). The fish was also reportedly introduced to Malili Lake system and Lake Poso in South Sulawesi where they severely impacted the population of endemic species through predation and competition (Herder et al. 2012; Sentosa & Wijaya 2012; Herder et al. 2022).

The Indonesian Government through Ministry of Fisheries and Marine Affairs have regulated distribution and banned several invasive species in the Minister Regulation No. 19/2020 which dictates that about 75 species of animals including fish, invertebrates, and amphibia were prohibited from distribution and entry in Indonesian territory (KKP 2020). The regulation was erected to control the circulation of potentially invasive species, and mitigate the introduction of non-native species that could harm endemic ecosystems. The list from the regulation includes several cichlid species such as *Cichlasoma trimaculatum*, now valid as *Amphilophus trimaculatus* (Fricke et al. 2022), *Amphilophus labiatus*, *Amphilopus citrinellus*, and *Mayaheros urophthalmus*, which are usually crossbred as flowerhorn varieties.

Flowerhorn breeding usually resulted in many morphological variations, and the designated species of the fish itself can be unclear resulting of generations of hybridization. Varied morphological forms and external characteristics would hinder identifying flowerhorn fish. Hence, relying exclusively on morphological analysis for identification is generally inadequate. A further step is needed to identify flowerhorn fish, especially in molecular level.

Molecular identification of cichlid fish using DNA barcoding method have been previously conducted with overall success (van der Bank

2019), and even successfully identify closely related species such as species of *Oreochromis* and *Coptodon* using *COI* marker (Panprommin et al. 2019). Mitochondrial *COI* gene marker has been widely used as universal barcode for fish identification and can be used for identification and monitoring of invasive species in general (Nagarajan et al. 2020). In Indonesia, DNA barcoding using *COI* marker has been successfully used to identify invasive *Amphilophus* cichlid species in Brantas River (Amin et al. 2019), which further assert that *COI* gene is generally robust for identification of cichlid fish. This study aimed to identify flowerhorn fish with various morphological types that were part of the ornamental fish trade. DNA barcoding method using *COI* marker was employed to ascertain the species of the distributed flowerhorn fish in Yogyakarta area.

MATERIALS AND METHODS

Sample Processing

Samples were collected in 2019 and 2020 during annual invasive species mapping held by Fish Quarantine Station in Yogyakarta. Six fish samples were obtained from various ornamental fish shops and patrons of the Yogyakarta Fish Quarantine Station. The fish samples were given sample voucher corresponding to their trade names: LH1CC (Cencu), LH2KF (Kamfa), LH3TS (Thai Silk), LH4KR (Kirin), LH5PR (Parrot), and LH6VJ (Vieja). Sample materials were taken by anesthetizing the fish followed by aseptically cutting 1 cm^2 of the caudal fin. DNA was extracted using FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen) with the steps according to the manufacturer's manual.

PCR Amplification

DNA barcoding was used to further identify each specimen by amplifying the *COI* gene using PCR. The PCR reaction were prepared using MyTaq PCR mix (Bioline), containing Taq DNA polymerase, dNTPs, $MgCl_2$ and buffer. Primer pair used in this study was COX1 F (TCA CAC GTT GAT TTT TCT CGA CT) and COX1 R (AAT AAG CGC GTG TGT CAA CG), self-developed using Primer 3 Plus software (Rozen & Skaletsky 2000). PCR process consisted of one cycle of pre-denaturation step at 94˚C for 3 minutes, followed by 35 cycles of denaturation step at 94˚C for 1 minutes, annealing at 50˚C for 30 seconds, and extension at 72˚C for 1 minute, and a final extension step at 72˚C for 5 minutes.

Amplified DNA was checked using gel electrophoresis with 1% agarose in 1X TBE. The gel was run in Mupid EXu Submarine Electrophoresis System at 10 V for 20 minutes and visualized using UV Transilluminator. Samples were sequenced by sequencing facility at 1st Base Asia (Singapore) using a sanger sequencing method.

Sequence Analysis

Raw sequences were processed post-sequencing before being aligned and analyzed. Sequences were checked and edited using BioEdit 7.2 (Hall 1999), and subsequently aligned using ClustalW 2.1 algorithm (Larkin et al. 2007). Nucleotide BLAST by NCBI was used for sequence identification. Phylogenetic analysis was conducted using MEGA11 software (Tamura et al. 2021). Tree-based analysis was performed using a Neighbor-Joining (NJ) method. The NJ phylogenetic tree was produced by bootstrap method with 1000 replicates to find the consensus tree. *COI* sequence of *Andinoacara rivulatus* (MT505734) was added as an outgroup. Pairwise genetic distances were calculated using Kimura-2 Parameter (K2P) using DnaSP ver.6 (Rozas et al. 2017).

RESULTS AND DISCUSSION

Results

Molecular Identification with BLAST Analysis

DNA sequences were amplified from partial *COI* gene. The sequences were compared to other *COI* genes in the GenBank database using BLAST algorithm. The result was selected and confirmed by higher percent identity score (95 - 100%), which concede with higher similarity from the GenBank sequences. Thus, 100% percent identity score would be interpreted as the sample and comparison sequence being the same species.

The NCBI BLAST and BOLD analysis results (Table 1) showed that all samples had 100% identity with *Amphilophus trimaculatus* (GenBank: MN888505; BOLD: MT975935). The BLAST results also had 100% query cover and zero e-value, meaning each queried sequence was fully aligned with the GenBank reference sequence with zero error value.

Table 2 shows the results of other congeneric and intergeneric species. *Amphilophus lyonsi* (MG936655) and *Amphilophus citrinellus* (MG496077) had the highest BLAST percent identity at 95.73% and 95.41%, respectively, followed by *Amphilophus labiatus* (JQ667489) at 95.40. The top two congeneric related species, according to BOLD species identifier, are *A. lyonsi* (DQ11919) and *A. citrinellus* (JN024798). The results indicated that the *COI* sequences from the six specimens were homologous to the *COI* DNA sequence of *Amphilophus trimaculatus*.

Table 1. Results of NCBI BLAST and BOLD Species Identifier based on *COI* sequence from the six flowerhorn samples.

No	Sample	GenBank Asc. no.	BLAST					BOLD		
			Species	Percent Identity	Query Cover	e-value	Top Similar	Species	Similarity	Top Similar
1	LH1CC	OQ371322	*Amphilopus trimaculatus*	100%	100%	0	MN888505	*Amphilopus trimaculatus*	100%	MT975935
2	LH2KF	OQ371323	*Amphilopus trimaculatus*	100%	100%	0	MN888505	*Amphilopus trimaculatus*	100%	MT975935
3	LH3TS	OQ371324	*Amphilopus trimaculatus*	100%	100%	0	MN888505	*Amphilopus trimaculatus*	100%	MT975935
4	LH4KR	OQ371325	*Amphilopus trimaculatus*	100%	100%	0	MN888505	*Amphilopus trimaculatus*	100%	MT975935
5	LH5PR	OQ371326	*Amphilopus trimaculatus*	100%	100%	0	MN888505	*Amphilopus trimaculatus*	100%	MT975935
6	LH6VJ	OQ371327	*Amphilopus trimaculatus*	100%	100%	0	MN888505	*Amphilopus trimaculatus*	100%	MT975935

Table 2. Top five related species results from BLAST and BOLD identification.

No	BLAST					BOLD		
	Species	Percent Identity	Query Cover	e-value	Asc. Number	Species	Similarity	Asc. Number
1	*Amphilophus lyonsi*	95.73%	99%	0	MG936655	*Amphilophus lyonsi*	95.73%	DQ119199
2	*Amphilophus citrinellus*	95.41%	99%	0	MG496077	*Amphilophus citrinellus*	95.55%	JN024798
3	*Amphilophus labiatus*	95.41%	99%	0	JQ667489	*Amphilophus labiatus*	95.52%	Unpublished
4	*Parachromis managuensis*	93.53%	100%	0	KU692739	*Amphilopus astorquii*	95.52%	Unpublished
5	*Panamius panamensis*	93.35%	99%	0	MG937088	*Amphilopus chancho*	95.52%	Unpublished

Pairwise Distance Analysis

Pairwise distance analysis estimates the evolutionary divergence between compared sequences. Analysis was conducted using Kimura 2-parameter (K2P) model. Results from pairwise distance analysis is presented in table 3 with percentage K2P values. Samples were compared with sequences derived from BLAST result and additional *Vieja* genus: *Vieja maculicauda* (MG496249), *Vieja fenestrata* (HM379818), and *Vieja melanura* with the valid name *V. melanurus* (MZ379719) for further comparison

Based from distance analysis, intraspecific genetic distance value was at 0.00%. Distance value of 4.95 – 21.25% showed inter-generic distance. All samples showed 0.00% genetic distance with *Amphilophus trimaculatus* sequence. Samples higher genetic divergence from the *Amphilophus* and *Vieja* genus sequences with 4.95% pairwise distance value and 13.52 – 14.29% value compared to the outgroup. It can be inferred that the six samples belong to the same taxonomic group as *Amphilophus trimaculatus* sequence.

Phylogenetic Tree Analysis

Tree analysis was conducted using Neighbor-Joining method. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (K2P). A total of 12 aligned and trimmed sequences were used to make the phylogenetic tree, each comprised of 634 bp. Six *COI* sequences were derived from our samples presented with coded label, while the rest were sequences derived from GenBank for comparison. All trees were rooted using outgroup *Andinoacara rivulatus* sequence. Evolutionary distance was calculated using Kimura 2-parameters on all trees with 1000 bootstrap replicates. Phylogenetic tree is presented in Figure 1.

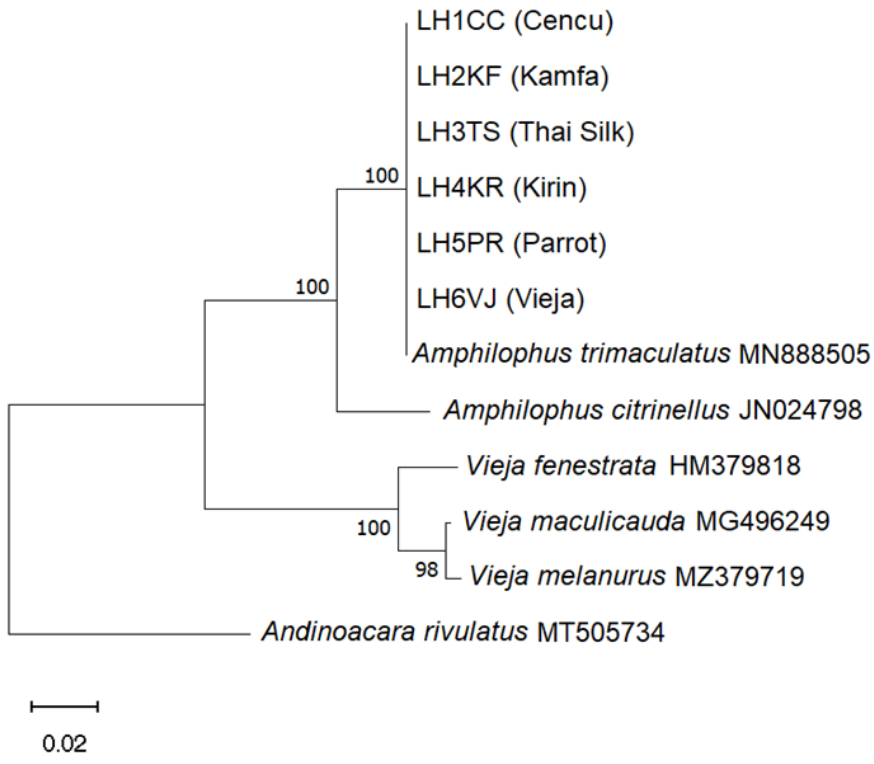

Figure 1. Phylogenetic analysis results using Neighbor-Joining (NJ). Bar represents calculated genetic distance based on the number of base substitutions for each site. Numbers on the branches indicated bootstrap support. Samples were coded with "LH-" prefix followed by a number and 2 letters of morphotype code.

Table 3. Genetic distance between samples and compared sequences (percentage of K2P).

No	Species	1	2	3	4	5	6	7	8	9	10	11
1	LH1CC (Cencu)											
2	LH2KF (Kamfa)	0.00										
3	LH3TS (Thai_Silk)	0.00	0.00									
4	LH4KR (Kirin)	0.00	0.00	0.00								
5	LH5PR (Parrot)	0.00	0.00	0.00	0.00							
6	LH6VJ (Vieja)	0.00	0.00	0.00	0.00	0.00						
7	*Amphilophus trimaculatus*	0.00	0.00	0.00	0.00	0.00	0.00					
8	*Amphilophus citrinellus*	4.95	4.95	4.95	4.95	4.95	4.95	4.95				
9	*Vieja maculicauda*	13.52	13.52	13.52	13.52	13.52	13.52	13.52	14.18			
10	*Vieja fenestrate*	13.90	13.90	13.90	13.90	13.90	13.90	13.90	13.95	3.93		
11	*Vieja melanura*	14.29	14.29	14.29	14.29	14.29	14.29	14.29	14.96	0.95	3.58	
12	*Andinoacara rivulatus*	19.36	19.36	19.36	19.36	19.36	19.36	19.36	20.50	21.25	20.82	21.25

The phylogenetic tree indicated that all six samples formed a distinct grouping with the *Amphilophus trimaculatus* sequence. *Amphilophus* and *Vieja* genera made a separate group from the sample sequences, thus inferred that they are genetically distant from *Amphilophus trimaculatus* group. The topology strongly suggests that all samples were the same species and classified as *Amphilophus trimaculatus* despite morphological difference.

Discussion

Flowerhorn, a hybrid breed of several cichlids has been known to have unclear taxonomic status. Morphological distinctions have led to the rise of several "variants" of flowerhorn that were circulated in the market such as "Zhen zhu" or “Cencu” variant with pearly-white scale coloration, "Golden base" variant characterized by yellow-colored body, and "Kamfa" variety with larger head bump. It is considered almost impossible to identify the original cichlid species used to make flowerhorn hybrid, let alone designate the taxonomic status. Hence, this study used molecular identification using *COI* gene in an effort to shed some light on the phylogenetics and taxonomic position of flowerhorn cichlid.

The family of Cichlidae or cichlid fishes was known to be highly adaptable and resilient to environmental changes. Previous work by van Rijssel et al. (2021) elucidated how a species of cichlid (*Haplochromis pyrrhocephalus*) showed morphological adaptation responses to environmental perturbation, which the morphological changes transcended species-exclusive characteristics. Such natural morphological adaptation remains unclear whether it was from hybridization, phenotypical plasticity or evolution. In case of flowerhorn species, morphological differences were exclusively artificial as a result of selective hybridization. There is currently limited evidence that the flowerhorn morphology would change when the fish was released outside the aquarium environment. However, in spite of hobbyists’ beliefs that domestication is able to tame the rapaciousness of flowerhorn, it is scientifically proven that flowerhorns can survive and thrive in natural environment, which accounts to their invasive capability (Mutia et al. 2007; Herder et al. 2012)

Previous studies such as Shirak et al. (2009) used partial sequence of mitochondrial *COI* to identify native and introduced cichlid fishes. Since then, the *COI* gene has also been used to identify and map invasive cichlid fish species in aquatic environment (Pèlèbè et al. 2021) and investigate the genetic diversity of the designated invasive species (De la Ossa -Guerra et al. 2020). Partial sequence of *COI* gene was also successfully amplified in this study and yielded 650 bp of nucleotide sequence for mo-

lecular identification. The main idea of the molecular identification process was to verify whether the flowerhorn aquarium specimens found in this study belonged to which cichlid species that were deemed invasive species, to which the culture and translocation are regulated by The Indonesian government.

Molecular identification process was initially performed by using NCBI BLAST and BOLD species identifier algorithm to investigate the percent identity between *COI* genes of the six specimens to other *COI* sequences in GenBank. The results showed that all sequences had 100% similarity with *Amphilophus trimaculatus* sequence, and to a lesser extent *Amphilophus citrinelus* and *A. labiatus*.

Further comparison with pairwise distance analysis showed that all six specimens had zero genetic divergence with *A. trimaculatus* sequence, while higher divergence was shown with *Amphilophus* with 4.95% genetic distance. The genetic distance with *Vieja* genus showed higher divergence at 13.52 - 14.29%. *Vieja* genus was also used as comparison since some members of the genus were often used as parent for cross-breeding flowerhorn fish (Nico et al. 2007). *Andinoacara rivulatus* or Green Terror fish, another possible parent breed for flowerhorn variant, was used as outgroup and showed 19.36% genetic distance compared to the specimens. The results of genetic distance exhibited homologous *COI* gene sequences from the six specimen and *A. trimaculatus*.

Phylogenetic analysis showed the six flowerhorn specimens clustered into one clade alongside *A. trimaculatus* sequence, while the other genus formed their own clade. The samples also indicated being the same haplotype as the *A. trimaculatus* sequence, being in the same end-point of the branch. The trees topology and phylogenetic position further assert that the flowerhorn samples can be identified as *A. trimaculatus*.

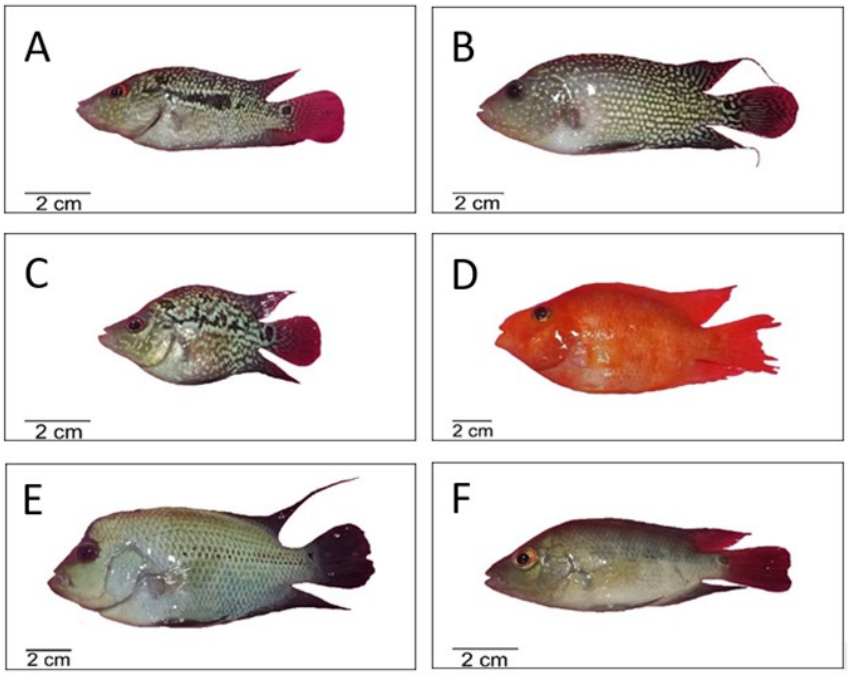

Figure 2. Samples with different morphotypes: (A) Cencu - LH1CC, (B) Kamfa - LH2KF, (C) Thai Silk - LH3TS, (D) Kirin - LH4KR, (E) Parrot - LH5PR, (F) Vieja - LH6VJ.

It is interesting to note that among morphologically distinct samples, especially dubbed as variants of flowerhorn species (Figure 2) such as Zhen Zhu, Kamfa, Parrot, Vieja, Kirin, and Thai Silk, molecular analysis classified them as one clade alongside *A. trimaculatus* with 0% genetic

divergence, while genus with potential parentage showed > 4% divergence as in the *Amphilopus* genus. Previous study by Wang et al. (2015) found that K2P genetic divergence among congeneric farmed freshwater fish averaged 5%, while conspecific divergence averaged 0.34%. The result is similar to this study, with intraspecific divergence is 0% and interspecific divergence being 4.95%.

Red devil fish (*Amphilopus labiatus*) and Midas cichlid (*Amphilopus citrinelus*) were known as potential cross to obtain specific variety of flowerhorn fish, as well as *A. trimaculatus* (Three spotted cichlid). Other cichlid fish such a *Cichlasoma festae*, now valid as *Mesoheros festae* (Guayas cichlid), and another hybrid cichlid called Blood Parrot cichlid were also suggested as cross-breeding material to create flowerhorn fish (Rahmati-Holasoo et al. 2015). However, it can be assumed based on the genetic divergence analysis that the parental lineage for the specimens could be *A. trimaculatus*.

The results of this study were entirely based on comparison and analysis between mitochondrial *COI* gene. It is generally accepted that mitochondrial inheritance in animals is uniparental, particularly in the matrilineal lineage. The cellular mitochondrion is inherited through the mitochondria of the oocyte, hence the mitochondrial genes would reflect the maternal origin of the individual (Sato & Sato 2013). The result of the species being closely related to *A. trimaculatus* in the molecular analysis may only reflect the maternal lineage of specimens.

We could not identify the parental hybrid by using just mitochondrial *COI* sequence for analysis, however our findings still raised a question regarding to the status of the flowerhorn specimen being a hybridized fish in this study. All samples were not collected from only one flowerhorn breeding facility, rather they came from various shops and patrons of The Fish Quarantine Station in Yogyakarta. Despite varied source and morphology, the mtDNA *COI* sequences from all six specimens did not exhibit polymorphism and had consistent genetic divergence value.

We also assume that the parental linkage is exclusively maternal. Although, the case of heteroplasmy or paternal leakage has been known to occur in hybridized fish. For instance, previous work by Morgan et al. (2013) found indication of paternal leakage in mitochondrial DNA of hybridized Scombrid fish. However, we took the general assumption that heteroplasmy or parental leakage does not occur in our *COI* sequence by the absence of double peaks and consistently similar nucleotide sequence between the sample sequences. Presence of double peaks in the nucleotide sequence of the *COI* region is an indicator of parental leakage in mitochondrial sequence (Rodríguez-Pena et al. 2020).

Phylogenetic analysis of mtDNA *COI* strongly suggested that the specimens were monophyletic with 100% similarity and 0% genetic divergence from *A. trimaculatus* sequence. All samples had identical *COI* sequence, despite being collected from various sources. Morphological differences were vastly distinctive between samples, despite molecular analysis found all to be one species of *A. trimaculatum*. Clear morphological distinction with the parent species was indeed a trait of hybridized fish (Selz et al. 2014; Pauers et al. 2018). In our case, different morphological variation of flowerhorns breed despite all specimen being conspecific with *A. trimaculatus* would infer that flowerhorn specimens in this study were solely bred from a flowerhorn variation of *A. trimaculatus* origin as opposed to being a hybrid or cross-bred from different cichlid species. However, regarding the verification of the fish being hybrid, our findings were inconclusive since mitochondria *COI* gene is inherited mat-

rilineally. The *COI* gene can be reliably used to identify and delineate cichlid species, but in the case of hybridization and hemiplasy, an integrative procedure is preferred (Breman et al. 2016). A more complex approach such as using *RAG1* nuclear gene (Kim et al. 2020), combination between mtDNA and nuclear *RYR3* gene marker (Qu et al. 2018), or multi-locus gene analysis with mitochondrial control region gene, nuclear gene, and microsatellite loci (Willis et al. 2012) or similar approach should be considered for future study to identify both parental hereditary components within their genome

The results of this analysis however did not diminish the fact that each specimen has the potential to be an invasive species if released in natural environment. The species of *Amphilophus trimaculatus* (previously named *Cichlasoma trimaculatum*) has been known as an invasive fish and their breed has been previously reported to invade local ecosystems in Indonesia, such as Lake Matano in Sulawesi (Nasution & Dina 2019). The fish was also reported as being invasive in Lake Sempor in Central Java (Hedianto et al. 2014) where the *A. trimaculatus* flowerhorn species dominated overall fish catch in the lake. Previously mentioned study also reported that fish is unpopular for consumption and fetch low price in the market compared local commodities like barb (*Barbonymus goniotus*), ceba fish (*Puntius binotatus*), and lunjar fish (*Rasbora argyrotaenia*). Nasution et al. (2022) reported *A. trimaculatum* population is relatively abundant in the littoral zone of Lake Mahalona, Sulawesi, consisted of up to 21.34% from the total population structure despite being an alien species. Reports outside Indonesia included India's Lake Chenai (Daniel et al. 2020) and River Cauvery (Kumar et al. 2020) where the fish exhibited predatory behavior and may deter predation by having spiny dorsal fins. The potential for flowerhorn fish to become an invasive species should be considered in the aquarium trade; as a result, fish distributors and owners should check the regulations and avoid releasing the fish into the local environments.

CONCLUSIONS

The flowerhorn fish samples with distinct morphological characteristics had homologous mtDNA *COI* sequence, and were conspecific to *Amphilophus trimaculatus* sequence according to phylogenetic and distance analysis. It is assumed that based on mtDNA *COI* all flowerhorn samples were bred from flowerhorn parents with *A. trimaculatus* origin. Further study should investigate the hybridization of different flowerhorn fish to confirm whether the fish were bred from different species parentals using other DNA markers, such as nuclear genes, microsatellites, or a combined approach. Our findings should also be used as precaution for the flowerhorn's potential as invasive fish species for being in the lineage of *A. trimaculatus*, a previously known invasive species.

AUTHOR CONTRIBUTION

D.W.K.S. designed the research, performed experiments, analyzed the data, and wrote the manuscript; H.A. and H.R collected the samples and analyzed data; H.B analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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