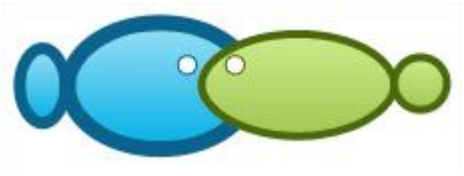

Genetic variation of domesticated discus (*Symphysodon* spp.)

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Abstract. Domesticated discus (*Symphysodon* spp.) exhibits highly diversified phenotypes appearances. However, genetic basis of the discus varieties and the relationship among them, that is fundamental and crucial for a proper management and breeding program, is currently unavailable. The present study focuses on the genetic relationship of the four wild types (Green, Heckel, Blue and Brown) and ten selected domesticated discus (Blue Diamond, Ghost, Golden, Snow White, Albino Red Cover, Red Snakeskin, Golden Tiger, Rose Red, Pigeon Blood and Leopard), inferred from the mitochondrial DNA cytochrome c oxidase subunit 1 and cytochrome b genes, and nuclear DNA recombination activating gene 1. Concatenated nucleotide sequences truncated to 3,242 base pair revealed that the Green discus was comparatively most differentiated (0.56–0.65%) from the other wild discus, while Blue, Brown and Heckel were closely related (0.03-0.22%). Specimens of the same group formed high bootstrap-supported clusters (> 77%) and were clustered with own members in the gene tree except Red Snakeskin and Rose Red, indicating occurrence of phenotypic ambiguity leading to misclassification of discus in fish farm. Rose Red was mutated from Brown discus. Pigeon Blood formed a separate cluster in the gene tree and differed genetically the most from all the others, suggesting a possible historical crossbreeding attempt using genetically distant parents. Genetic homogeneity was observed among Blue Diamond, Ghost, Golden, Snow White, Albino Red Cover, Red Snakeskin and Leopard, with all these varieties linked closely to Heckel, Blue and Brown, indicating these are the likely genetic origin for these varieties. This finding also revealed discus flexibility in developing progeny with diverse physical appearances. High phenotypic variability in the absence of genetic variants may be associated with adaptive phenotypic plasticity in discus to better adapt in novel environments. The results presented herein suggest an inclusive fitness advantage for inbreeding individuals in discus, as part of the biparental care strategy in Cichlid fishes. Our study demonstrated that a genetic-based classification for selective breeding in tank-bred discus is optional, while a systematic classification using phenotypic features such as background colour, body markings and other traits for breeding management, instead is more relevant and economical for discus breeders/enthusiasts worldwide.
Key Words: discus *Symphysodon*, genetics, inbreeding, mitochondrial DNA, varieties.

Introduction. Ornamental fish trade is a multibillion-dollar industry with more than 2,500 species of fish traded globally (Dey 2016). Of these, 60% of the ornamental fishes are freshwater origin, with the most popular ones include live bearers, tetras, angels, goldfish, danios and discus (Dey 2016). Malaysia is among the top producers of ornamental fish in the world, with export values surpassing RM340 million (USD 82 million) in 2015. In this context, the discus *Symphysodon* spp. represents a popular ornamental fish, with 54 varieties produced in Malaysia for global export (Ng 2004). Domesticated discus with high colour variations and pattern markings are in great demand by freshwater aquarium enthusiasts and command high prices worldwide.

Discus originates from the Amazon River basin in South America and those occurring in the natural habitat are considered as wild type or principle phenotype. Discus

produced in captivity is known as tank-bred fish and is domesticated for ornamental purposes. Colours and patterns are important phenotypes for discus and other aquarium fish because they will determine its popularity and market value. New discus varieties are developed by fish breeders to meet the market requirements either naturally or by outcrossing or inbreeding of wild types (Koh et al 1999; Ng 2004). Wild discus was initially recognized based on their physical appearance and distribution within the Amazon tributaries (Farias & Hrbek 2008). The typical wild type discus was assigned into Blue, Black (Heckel), Brown, Green and Abacaxi, in concordance to their allopatric distribution (Amado et al 2011). Later, by integrating molecular techniques, sympatric discus was found to have shared nuclear alleles and mitochondrial haplotypes (Ready et al 2006; Farias & Hrbek 2008; Amado et al 2011) suggesting evolutionary events such as genetic and ecological exchangeability, historical admixture, introgressive hybridization (Amado et al 2011) and/or undergoing process of diversification (Farias & Hrbek 2008). Three valid discus species are currently recognized, *i.e*. *S. discus*, *S. tarzoo* and *S. aequifasciata* (Froese & Pauly 2019). Latest molecular study of the discus inferring from the full mitochondrial genomes similarly indicated a close kinship among them, evident by genetic similarity 98 to 99%, and genetic distance ranges from 0.25 to 1.39% (Li et al 2016a, 2016b).

Domesticated discus was produced using trial and error methods (Koh et al 1999) via repeated cross-, back-cross and/or inbreeding over multiple generations, most of which resulted in a loss of track on the breeding line due to poor production documentation. Discus varieties exhibit highly diversified phenotypes appearances and are classified solely according to colour variance and other features, but the genetic basis underlying these phenotypes is poorly known. Understanding the phylogenetic relationship of the wild and domesticated discus is essential for a proper management and breeding program, to preserve genetic variability and prevent inbreeding depression that is likely to result from the current unplanned breeding practices in discus (Koh et al 1999). As such, research on genetic relationships among the broodstock (wild type) and tank-bred discus is therefore fundamental and crucial. Scientific classification for all existing discus varieties that can be accepted worldwide is also necessary, as it may facilitate the implementation of such programs and the accurate exchange of information among breeders (Koh et al 1999).

Previous studies on domesticated discus were directed to breeding, larval rearing and development (Önal et al 2010; Mattos et al 2015; Satoh et al 2017; Liu et al 2019), and a single report on the genetic assessment among the wild and domesticated discus (Koh et al 1999). Koh et al (1999) evaluated the four wild types and five cultivated discus varieties using Random Amplified Polymorphic DNA (RAPD) fingerprinting method, concluding that the gene pool of the tank-bred discus is smaller than that of the wild forms, the Heckel discus is genetically the most divergent to the other wild forms and *S. aequiefasciata* (wild Green, Blue and Brown) is likely the genetic origin of the tank-bred varieties (Turquoise, Pigeon, Ghost, Cobalt and Solid Red). Molecular markers have been widely used and proven powerful in detecting genetic uniqueness of individual species or populations, to answer questions related to taxonomy, ecology, forensic science, evolution, conservation and management of natural resources and genetic improvement programs (Askari et al 2013; Tan et al 2015, 2016, 2019, 2020).

In this study, the phylogenetic relationship of four wild types and ten selected discus varieties was explored, this time using a more advanced and reliable method than previously described (Koh et al 1999). Outcomes generated from this study are necessary to underscore the genetic basis for scientific classification of discus varieties, a vital step in the establishment of selective breeding programs.

Material and Method

Ethical statement. Fish samples were captured from its aquarium using hand net, and anaesthetized using small amount of Tricaine mesylate (MS-222) prior to photographing and clipping of small portion of the caudal fin rays. The fish was released back to its aquarium and allowed to recover for several weeks until the caudal fin regrow fully. The

species is not in the IUCN list of endangered or protected species. No further experiment was performed on the fish.

Sample collection and preservation. Four principle phenotypes (two specimens each) and ten domesticated discus varieties (five specimens each) (*Symphysodon* spp.) were collected from Aquacity Tropical Fish, Kuala Lumpur, Malaysia. Identity of each discus was determined according to the Discus Catalogue (Ng 2004) and interview with the farm's owner. The principle phenotypes used in this study are Green, Blue, Brown and Heckel, while the varieties are Blue Diamond, Pigeon Blood, Ghost, Golden, Snow White, Albino Red Cover, Red Snakeskin, Golden Tiger, Rose Red and Leopard. A small portion of the caudal fin rays were cut from each individual and preserved in 1.5 mL microcentrifuge tubes containing 95% ethanol. Fish was released back into its original tank to recuperate. Fin tissues were stored at the Genomic Laboratory, Institute of Marine Biotechnology (IMB), Universiti Malaysia Terengganu at room temperature (~20°C) until further use.

Genomic DNA isolation and polymerase chain reactions (PCR) amplification. Total genomic DNA was isolated from fin tissue using salt extraction method (Aljanabi & Martinez 1997). The PCR amplifications were conducted for mitochondrial DNA (mtDNA) cytochrome c oxidase subunit 1 (CO1), cytochrome b (CYTB), and nuclear DNA (nuDNA) recombination activating gene 1 (RAG1), following PCR set-up and thermal cycling protocols but with modifications after Ward et al (2005), Farias & Hrbek (2008) and Ready et al (2006), respectively (Table 1). PCR products were visualized on a 1.7% agarose gel stained with SYBR Safe to confirm successful amplifications. All products were sent for sequencing (Apical Scientific Laboratories Sdn. Bhd., Selangor Malaysia) and reading from both DNA strands for all markers.

Data analysis. Sequences were aligned and edited using ClustalW implemented in MEGA X (Kumar et al 2018). DNA sequences were verified for correct identity by using Basic Local Alignment Search Tool (BLAST) in NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) before further analyses. Aligned sequences were checked and analysed for nucleotide/amino acid polymorphisms, including heterogeneous base composition and insertion/deletion site. Unique sequences (haplotypes) of each discus group were deposited into GenBank under accession numbers MV167084-167100 (CO1), MW174803-174820 (CYTB) and MV201877-201908 (RAG1).

Preliminary investigation on tree topology of individual's gene marker returned similar phylogram, thus sequences of all markers were concatenated for phylogenetic analysis. Gene tree was constructed using the maximum likelihood (ML) method in MEGA X, applying 1,000 bootstraps replications for the confidence levels at each branch node (Hall 2013). Hasegawa-Kishino-Yano + gamma distribution (HKY+G) (Hasegawa et al 1985) was determined as the best nucleotide substitution model in MEGA X. Pairwise genetic distance among individuals/groups was determined in MEGA X.

Table 1

Modified PCR optimizations for *Symphysodon* spp. using the COI, CYTB and RAG1 markers

No.	*Marker*	*Primer name: sequence (5' to 3')*	*PCR set-up*	*Thermal cycling protocol*	*Reference*
1	CO1	FishF2: TCGACTAATCATAAAGATATCGGCAC FishR2: ACTTCAGGGTGACCGAAGAATCAGAA	50-100 ng of genomic DNA, 0.5 µL each primer, 12.5 µL Mytaq (Bioline) and 9.5 µL sterilized ultrapure water (ddH_2O) in total of 25 µL reaction mix.	Initial denaturation at 94ºC for 5 minutes (min) followed by 32 cycles of 94ºC for 30 seconds (sec), 55ºC for 30 sec, 72ºC for 1 min, final extension of 72ºC for 5 min and final hold at 4ºC.	Ward et al (2005)
2	CYTB	FISCYTB-F: ACCACCGTTGTTATTCAACTACAAGAAC TRUCCYTB-R: CCGACTTCCGGATTACAAGACCG	50 ng of DNA template, 10 µL Mytaq (Bioline), 0.6 µL each primer and 7.8 µL sterilized ultrapure water (ddH_2O) in total of 20 µL reaction mix.	Initial denaturation at 94ºC for 2 minutes (min), followed by 10 cycles of 94ºC for 30 seconds (sec), 50ºC for 40 sec, 72ºC for 90 sec, continued with another 30 cycles of 94ºC for 30 sec, 54ºC for 40 sec, 72ºC for 90 sec, final extension of 72ºC for 10 min and final hold at 4ºC.	Ready et al (2006)
3	RAG1	Rag1.HB.F.L1: CCWGCTGTITGYYTGGCCATIMG Rag1r.6: TGYTTTCAGCACGTCCTCCARYTC	50 ng of DNA template, 10 µL Mytaq (Bioline), 0.6 µL each primer and 7.8 µL sterilized ultrapure water (ddH_2O) in total of 20 µL reaction mix.	Initial denaturation at 94ºC for 2 minutes (min), followed by 10 cycles of 94ºC for 30 seconds (sec), 45ºC for 40 sec, 72ºC for 90 sec, continued with another 30 cycles of 94ºC for 30 sec, 51ºC for 40 sec, 72ºC for 90 sec, final extension of 72ºC for 5 min and final hold at 4ºC.	Farias & Hrbek (2008)

Results and Discussion. Eight and 49 individuals of principle phenotypes and tank-bred discus (*Symphysodon* spp.) were respectively sequenced at mtDNA COI and CYTB genes, and nuDNA RAG1 gene, with final length truncated to 650, 1,139 and 1,453 base pair (bp), respectively. BLAST analysis showed that our sequences matched with genus *Symphysodon* and multiple species of *Symphysodon*, thus *Symphysodon* spp. was used throughout the manuscript. The final sequence alignment of each gene marker revealed a total of 17 (CO1), 46 (CYTB) and two polymorphic sites (RAG1), resulting in synonymous substitutions in CO1 but 14 and one amino acid substitutions in CYTB and RAG1, respectively. Altogether, seven putative haplotypes were respectively obtained from COI and CYTB sequences, while five unique RAG1 sequences were obtained. Concatenated CO1, CYTB and RAG1 sequences with final length of 3,242 bp reveal ten unique haplotypes. Individuals within each discus group were genetically homogenous except for Blue, Golden Tiger and Pigeon Blood, where two substitution sites were detected in Blue while one mutation site respectively in Golden Tiger and Pigeon Blood. These results translated to the within group genetic divergence of 0.00 in all discus groups except 0.06, 0.02 and 0.02% in Blue, Golden Tiger and Pigeon Blood, respectively.

Specimens of the same group formed high bootstrap-supported clusters (> 77%) and were clustered with own members in the ML tree except Red Snakeskin and Rose Red (Figure 1). This reflects a relatively higher genetic distance observed within Rose Red (0.17%) and Red Snakeskin (0.75%), both pointing to the possibility of misidentification due to phenotypic ambiguity (Koh et al 1999). Blue Diamond, Ghost, Golden, Snow White, Albino Red Cover, Red Snakeskin and Leopard are genetically identical, indicating a common source of broodstock and lack of a genetic differentiation for the present phenotypic classification of the cultivated varieties (Koh et al 1999). Green discus is comparatively distinct from the other three principle phenotypes (0.56-0.65%), whereas Blue, Brown and Heckel are closely related (0.03-0.22%) (Table 2), observation similar to Ready et al (2006) and Farias & Hrbek (2008), but in contrast to Koh et al (1999).

Blue Diamond, Ghost, Golden, Snow White, Albino Red Cover, Red Snakeskin, Golden Tiger and Leopard formed a monophyletic cluster with Heckel, Brown and Blue discus (Figure 1), suggests that they are the likely genetic origin of these tank-bred varieties (Table 2). These findings also indicated discus versatility in producing progeny with multiple physical appearances. Further study involving other domesticated discus varieties is required to support this assumption and to uncover the potential of other principle phenotypes. On the other hand, Rose Red was a mutant form of the Brown discus (Figure 1). Pigeon Blood (and two phenotypically identified as Red Snakeskin) forms a separate cluster in the ML tree and is the most distantly related from all the others (0.76-1.29%, Table 2, Figure 1), suggesting a probable historical crossbreeding attempts between genetically distant parents. In this case, Green discus was possibly paired with either Heckel, Blue or Brown discus.

The intra-wild form and intra-domesticated genetic variability may be under- or overestimated due to the small sample sizes used (2-5 specimens). Since our study focused on the relative comparisons among the wild and domesticated discus and not the absolute genetic variation in the wild forms nor the cultivated varieties, the result herein, however, could only be more comprehensive if a higher sample size was available. Inbreeding depression has been avoided in many of the captivated breeding programs due to negative effects to the fitness related traits (Smallbone et al 2016). However, it is interesting to observe healthy traits (personal comm.) including the high phenotypic variations in the tank-bred discus populations despite the absence to low genetic variability among certain individuals. This may be associated with the adaptive phenotypic plasticity with fixed genotypic differences in discus as strategies for adaptation to novel environment. However, the underlying mechanism is yet to be unraveled. Current result suggests an inclusive fitness advantage for inbreeding individuals (Kokko & Ots 2006), as observed in the African cichlid (*Pelvicachromis taeniatus*) (Thünken et al 2007). Being biparental brood carer, both sexes of *P. taeniatus* prefer mating with unfamiliar close kin over non-kin, as biparental care, requiring synchronous behaviour between parents, is costly and any conflict between parents over care will reduce the fitness of the offspring (Thünken et al 2007). Related parents are

expected to be more cooperative and invested more than unrelated parents, thus inbreeding is an advantageous strategy in *P. taeniatus* (Thünken et al 2007). This may also apply to the discus fish with biparental care behaviour, hence, outcrossing of the discus fish to avoid inbreeding depression may not be a concern. To further support this, molecular data together with breeding performance of more tank-bred discus fish is therefore required.

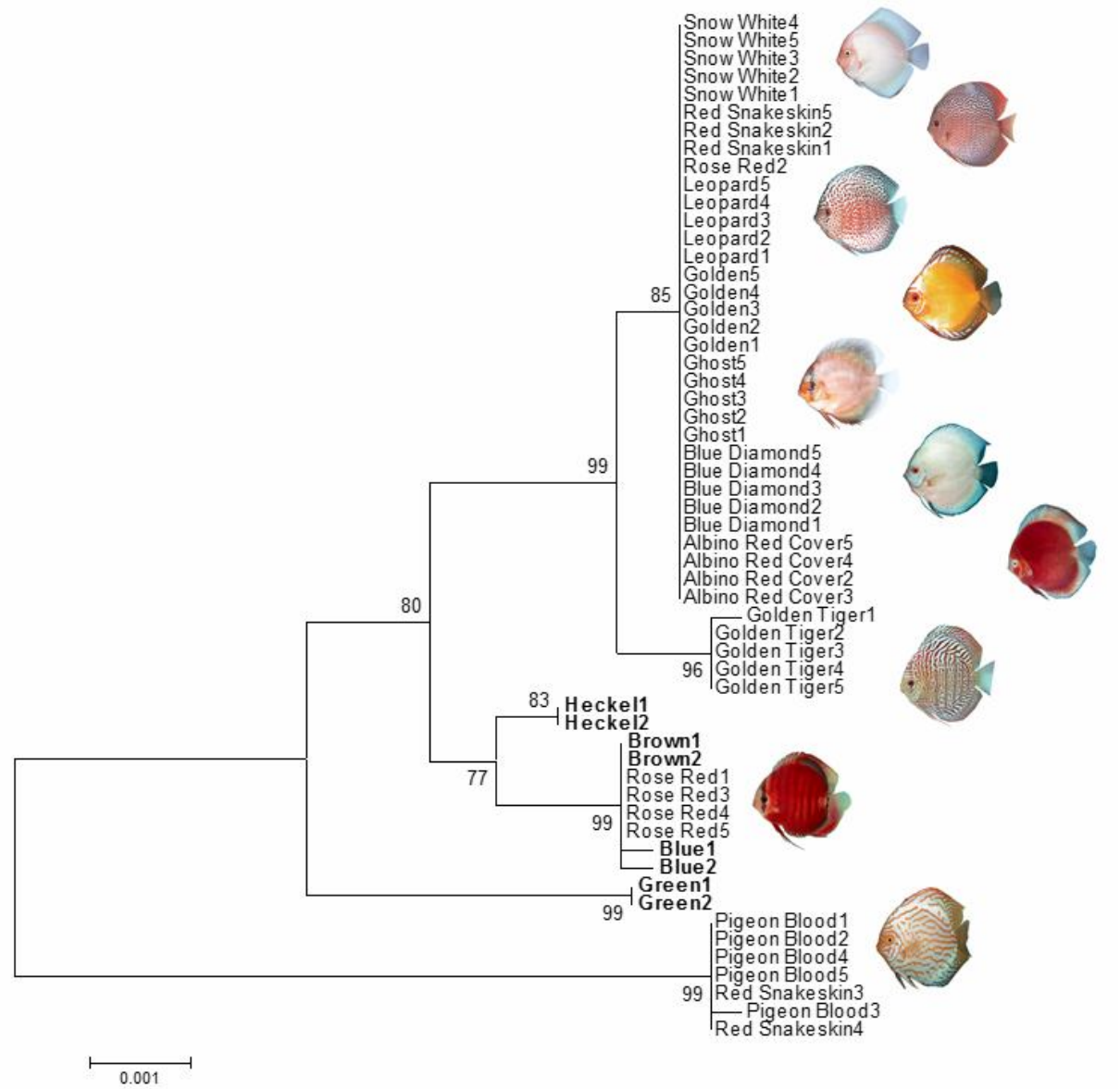

Figure 1. Scaled maximum likelihood gene tree of *Symphysodon* spp. inferred by the concatenated CO1, CYTB and RAG1 sequences (3,242 bp). The principle phenotype discus was **bold**.

Table 2

Genetic distance among discus (*Symphysodon* spp.) from Malaysian fish farm inferred by the concatenated CO1, CYTB and RAG1 sequences

		Principle phenotype				*Domesticated varieties*								
		Green	Blue	Brown	Heckel	Albino Red Cover	Blue Diamond	Ghost	Golden	Leopard	Pigeon Blood	Rose Red	Golden Tiger	Red Snakeskin
Principle phenotype	Green													
	Blue	0.0065												
	Brown	0.0062	0.0003											
	Heckel	0.0056	0.0022	0.0019										
Domesticated varieties	Albino Red Cover	0.0068	0.0047	0.0043	0.0037									
	Blue Diamond	0.0068	0.0047	0.0043	0.0037	0.0000								
	Ghost	0.0068	0.0047	0.0043	0.0037	0.0000	0.0000							
	Golden	0.0068	0.0047	0.0043	0.0037	0.0000	0.0000	0.0000						
	Leopard	0.0068	0.0047	0.0043	0.0037	0.0000	0.0000	0.0000	0.0000					
	Pigeon Blood	0.0126	0.0129	0.0125	0.0107	0.0125	0.0125	0.0125	0.0125	0.0125				
	Rose Red	0.0063	0.0012	0.0009	0.0022	0.0035	0.0035	0.0035	0.0035	0.0035	0.0125			
	Golden Tiger	0.0071	0.0050	0.0047	0.0040	0.0015	0.0015	0.0015	0.0015	0.0015	0.0129	0.0040		
	Red Snakeskin	0.0091	0.0079	0.0076	0.0065	0.0050	0.0050	0.0050	0.0050	0.0050	0.0076	0.0071	0.0060	
	Snow White	0.0068	0.0047	0.0043	0.0037	0.0000	0.0000	0.0000	0.0000	0.0000	0.0125	0.0035	0.0015	0.0050

Conclusions. The current study highlights the versatility of the Heckel, Blue and Brown discus in producing progeny with diverse phenotypic traits but fixed genetic mutation, possibly associated with adaptive phenotypic plasticity to adapt to the novel environment. Current result also suggests for an inclusive fitness advantage for inbreeding individuals in discus, as part of the biparental care strategy in Cichlid fishes. Our study demonstrates that a genetic-based classification for tank-bred discus is optional, and a systematic classification using phenotypic features such as background colour, body markings and other traits is indeed more relevant and economical for discus breeders/enthusiasts worldwide. The mitochondrial and nuclear markers are useful in evaluating genetic variation and inter- and intra-relationship of the wild and tank-bred discus. However, the markers could neither distinguish gene pool of the close kin nor used to estimate the phenotypic outcome of the offspring. Perhaps, future studies should aim to unravel the evolution and underlying mechanism related to pigmentation mutation in discus using transcriptomes or whole genome approaches.

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