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GENETIC IDENTIFICATION OF SELECTED ORNAMENTAL FISHES IN SEREMBAN, NEGERI SEMBILAN

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ABSTRACT

The ornamental fish sector is a widespread and global component of international trade, fisheries, aquaculture and development. The utilization of multiple trade names causes problem in species identification. Moreover, unmanaged trading could lead to severe threats to biodiversity. In this regard, DNA barcoding could effectively clarify the divergence of the species. Considering the utility of DNA barcoding as a comprehensive system for species identification and discovery, this study aims to investigate the genetic relationship and to construct the phylogenetic tree among those selected fish species collected from selected pet stores in Seremban, Negeri Sembilan. The 642bp barcode fragment of the Cytochrome c oxidase I (*COI*) gene was PCR amplified. Results from BLAST showed all the generated sequence were subjected to high percentage identity index and similarity between 99% to 100%. It was then analyzed using MEGA 7.0 through Neighbour-Joining (NJ) clustering and K2P distance-based approach. The analysis revealed straightforward identification of eight specimens into five species with increasing value of genetic distances from conspecific (0.05%) to the taxonomic level (20.18%). The phylogenetic analysis consists of own sequences and reference sequences obtained from the GenBank. All the specimens from different genus was found with high bootstrap value (n>90%) through Neighbour-Joining (NJ) and Maximum Likelihood method. Thus, DNA barcoding reflects the efficacy of the techniques in identifying the genetic assessment in selected ornamental fishes.

Keywords: species identification, ornamental fish, DNA barcoding, *COI* gene

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INTRODUCTION

Ornamental fishes are well known as aquarium fishes. According to Singh (2005), ornamental fishes are the most popular pets in the world. In addition, the popularity of aquarium keeping has been increasing over the years. There is a growing on demand for aquarium fishes in the domestic and international market and it has achieved high economic value across the globe which leads to increased growth in international fish trade as noted by Alam *et al*. (2016). The ornamental fishes were exported around the world with generic names or trade names such as “Barbs and “Carps” rather than proper zoological nomenclature (Dhar and Ghosh, 2015). This eventually creating difficulty in species identification.

In this scenario, DNA barcoding was proved to be the best approach for species identification and it turned to be helpful in studying biodiversity that were faced with uncertainty, like fish (April *et al*., 2011; Laskar *et al*., 2013), freshwater fish (Ward *et al*., 2005; Valdez-Moreno *et al*., 2009) and aquarium fish (Steinke *et al*., 2009; Collins *et al*., 2012; Dhar and Ghosh,2015). Balakrishnan (2005) emphasized the importance of linking the correct scientific names with the groups of individuals in order to define the priorities in biodiversity conservation.

There are many species can be identified by matching their DNA sequences with standard references

in a database (Espiñeira *et al*., 2008; Ogden, 2008). The cytochrome oxidase subunit I (*COI*) gene acts as a barcode, which is universal and suitable for most of the species (Hebert *et al*., 2003). Ward *et al*. (2009) stated that thousands of specimens had been generated using *COI* gene and are available in public database (GenBank and BOLD). Thus, the concrete reference database further proven the reliability of DNA barcoding in confirmation of species. Considering the promising use of DNA barcoding to identify species, this study aims to generate a comprehensive barcode reference library for freshwater ornamental fishes from Seremban, Negeri Sembilan. This finding will help future researchers and others for easy access of the ornamental fishes of this region and further action such as conservation can be implemented.

METHODS

Samples Collection

A total of 14 ornamental fish specimens were obtained in pet stores located in Seremban. In this study, two samples for each seven different species of freshwater ornamental fishes (Red Swordtail, Tiger Barb, Silver Shark, Goldfish, Siamese Fighting Fish, Black Molly and Altum Angel) were examined. The samples were collected and stored at -20°C in Molecular Laboratory of Universiti Teknologi MARA, Kuala Pilah.

DNA extraction, PCR amplification and DNA sequencing

Tissue samples were dissected from the body muscle and genomic DNA was extracted following the instructions of the commercial Wizard® Genomic DNA Purification Kit Manual (Promega, USA). A 650bp fragment of the mitochondrial COI gene was amplified in a thermocycler (CS cleaver, UK), using primer FishF2 (5' TGTAAAACGACGGCCAGTCGACTAAT 3') and FishR2 (5' CAGGAAACAGCTATGACACTTCAGGG 3') (Ward *et al*., 2005). The amplification reaction was performed in a total volume of 25µl for all the samples including 5µl 10x Green GoTaq buffer (Promega, USA), 1µl Primer Fish2 Forward, 1µl Primer Fish2 Reverse, 0.5µl dNTP mix (Promega, USA), 1.4µl MgCl2 (Promega, USA), 0.7µl DNA polymerase (Promega, USA), 4µl DNA template and 12.4µl ddH20. The PCR condition was carried out followed by 35 cycles for each steps: initial denaturation at 94ºC for 2 minutes, denaturation at 94ºC for 30 seconds, annealing at 57.9ºC for 40 seconds, extension at 72ºC for 45 seconds and final extension at 94ºC for 10 minutes followed by a hold at 4ºC. The PCR-amplified products were visualized on agarose gels (Next Gene Scientific, Malaysia). Gel product were viewed and documented via gel documentation system (Uvitec, UK). Sequencing was continued with successful PCR products. The DNA sequencing service was performed by First Base Laboratory Sdn.Bhd. Malaysia.

Data Analysis

The DNA sequences obtained from First Base Laboratory were submitted to BOLD system (http://www.boldsystems.org/) under a project named "DNA Barcoding of Ornamental Fishes" and to BLAST of GenBank for species identification. The sequences result was aligned with the reference sequences (obtained from the GenBank). The accession number of the specimens are from MK533710 to MK533717. The multiple sequence alignments and editing for the forward reactions were done using the CLUSTAL X program (Larkin *et al*., 2007) and subsequently aligned by eyes. CLUSTAL X is a multiple alignment program for DNA or protein. It can produce multiple sequence alignments of divergent sequences. The sequences had been aligned with the standard sequences and were trimmed to obtain the targeted length of 650 and the final alignment were saved to allow the data to proceed for genetic distance calculation and tree construction (Kumar *et al*., 2004). BioEdit software provided basic functions for protein and nucleic sequence editing, alignment, manipulation and analysis (Hall *et al*., 2011). The Neighbor-joining (NJ) trees and Maximum-likelihood (ML) tree of Kimura two-parameter (K2P) distance were used to provide a graphic representation of the pattern of divergence between species (Saitou and Mei, 1987). The 1000 bootstrap replications were performed in MEGA 7.0 software (Kumar *et al*., 2004). Ward *et al*. (2005) claimed that the K2P genetic distances are for defining the species, genus and family levels. MEGA 7.0 software is to construct the phylogenetic tree

based on the genetic distance. The purpose of the phylogenetic tree being constructed is to study the diversity of the selected ornamental fishes.

RESULT AND DISCUSSION

Amplification of the PCR products

The *COI* gene amplification of the 14 samples, have resulted in eight successfully amplified samples and were sent for the sequencing process (Figure 1). The non-amplified samples show no product from the beginning even after repeated PCR optimization process, and it was believed due to the degradation of the DNA caused by inaccurate handling of the samples. This is supported with a study conducted by Fogelström (2015).

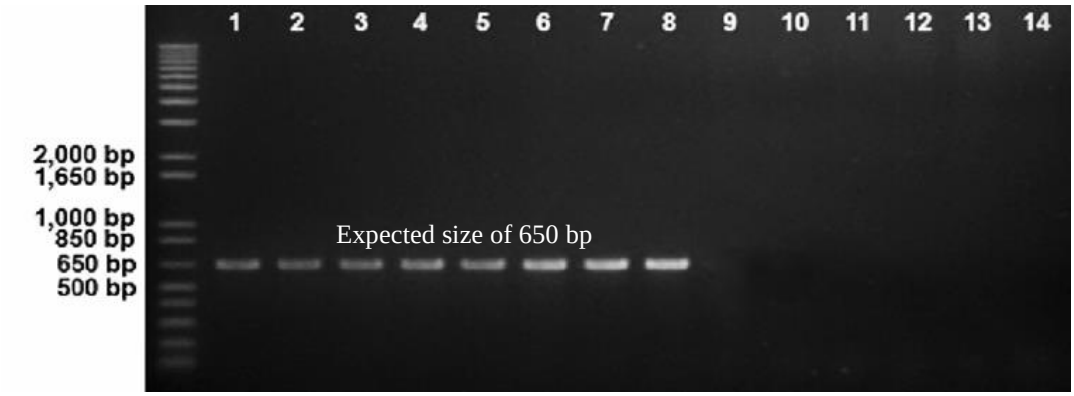

Figure 1. An image of a 1% agarose gel with DNA ladder 1kb, showing the separation of the PCR amplification products approximately 650bp in size.

Note:
Lane 1 : WFASW1
Lane 2 : WFABS1
Lane 3 : WFABS2
Lane 4 : WFATB1
Lane 5 : WFASS1
Lane 6 : WFASS2
Lane 7 : WFAGF1
Lane 8 : WFAGF2
Lane 9 till 14: Non-amplified samples

Species identification by pre-existing sequences in the database

The sequences undergo species validation through BLAST program in order to find the best match with the reference *COI* sequences from the GenBank and BOLD database. The DNA barcode database presents a system in identifying species based upon the discovering of the nearest match of the own sequences with database sequences. The extensive species identification based on the consensus of the similarity match on GenBank for the studied ornamental fish specimen revealed straightforward identification for eight specimens belonging to five species (Table 1).

Table 1. Closest match in the identification of the ornamental fish specimens from selected pet stores in Seremban, Malaysia.

Sample Code	Common Name	Close match in GenBank	Close match in BOLD
WFASW1	Red Swordtail	*Xiphophorus helleri* (100)	*Xiphophorus helleri* (100)
WFABS1	Fighting Fish	*Betta splendens* (99)	*Betta splendens* (100)
WFABS2	Fighting Fish	*Betta splendens* (99)	*Betta splendens* (100)
WFATB1	Tiger Barb	*Puntigrus tetrazona* (100)	*Puntigrus tetrazona* (100)
WFASS1	Silver Shark	*Balantiocheilos melanopterus* (100)	*Balantiocheilos melanopterus* (100)

WFASS2	Silver Shark	*Balantiocheilos melanopterus* (100)	*Balantiocheilos melanopterus* (100)
WFAGF1	Goldfish	*Carassius auratus* (100)	*Carassius auratus* (100)
WFAGF2	Goldfish	*Carassius auratus* (100)	*Carassius auratus* (100)

All the sequences were identified respective to their expected species with 99% and 100% similarity with GenBank sequences which confirmed that all the sequences were 99.999% accurate.

DNA sequence assignment

Distance matrix were performed using Kimura 2-parameter model (Kimura, 1980). The resulting matrix of 642bp containing 386 of conserved sites, 256 of variable sites and 237 of parsimony informative characters were detected. The results did not found any evidence of stop codons, insertions or deletions which represents functional mitochondrial *COI* gene fragment. The sequences were then submitted to Barcode of Life Data System (BOLD) for gaining their own Barcode Index Number (BINs) and accession number from GenBank (Table 2).

Table 2. List of DNA sequences from five species with their assigned BINs and GenBank accession number.

Family	Species	Sample ID	BINs	GenBank Accession Number	Size (bp)
Cyprinidae	*Puntigrus tetrazona*	WFATB1	WFA008-18	MK533710	642
Cyprinidae	*Balantiocheilos melanopterus*	WFASS1	WFA005-18	MK533714	642
		WFASS2	WFA006-18	MK533715	642
Cyprinidae	*Carassius auratus*	WFAGF1	WFA003-18	MK533716	642
		WFAGF2	WFA004-18	MK533717	642
Osphronemidae	*Betta splendens*	WFABS1	WFA001-18	MK533712	642
		WFABS2	WFA002-18	MK533711	642
Poeciliidae	*Xiphophorus helleri*	WFASW1	WFA007-18	MK533713	642

Ornamental fish COI gene assignment

Overall mean nucleotide frequencies for own specimens were G(17.53%), C(26.06%), A(25.98%) and T(30.42%). The base composition analysis of the COI sequence revealed AT content (52.34%) that was higher than GC content (47.66%). According Ros *et al*. (2007) on average across all taxa, the AT content was 75% (32%A, 43%T, 11%C, and 14%G). This high AT content is a general feature of the *COI* region in and is comparable to other studies on insect, athropods and mite taxa (Lunt *et al*. 1996; Navajas *et al*. 1996b).

The result from the distribution of sequence divergence showed there are three taxa (Perciformes, Cypriniformes and Cyprinidae) and some species belong to the same taxa with a low sequence divergence within species ranged from 0% to 0.16% (mean=0.05) (Table 3).

Table 3. Genetic divergence of Kimura-2-Parameter (K2P) at different taxonomic level for ornamental fish specimens from Seremban, Negeri Sembilan.

Comparison within	Taxa	Comparison	K2P genetic divergence (%)			
			Minimum	Mean	Maximum	SE
Species	3	3	0.00	0.05	0.16	0.02
Genus	0	0	0.00	0.00	0.00	0.00
Family	1	8	16.78	20.18	24.41	0.43

Within the genus, the results showed 0% which indicated no increase in genetic variability relative to species from a single region. Whereas in higher taxonomic level, the genetic divergence increased from 16.78% to 24.41% (mean=20.18) between genera within family which strengthen the fact that genetic changes had occurred. The same condition also happened to Shen *et al*. (2016), they obtained an increasing value in genetic distances of Cyprinidae fishes which are 0.36% for conspecific, 7.08%

for congeneric and 19.67% at taxonomic level. Thus, the result of the study consensus with the requirements stated in the BOLD system, it is essential to have a low sequence divergence within species compared to sequence divergence at higher taxonomic levels (Ward, 2009).

The summary of the barcode gap was done by using MUSCLE alignment program (Edgar, 2004) and Kimura 2-Parameter (Kimura, 1980). The mean intra-specific distances showed the minimum and maximum divergence of 0% with a mean divergence value of 0.03%. This is supported with a study conducted by Hebert *et al*. (2003) saying that the intraspecific genetic distances based on K2P are usually low (below 1%) and are rarely greater than 2% across a broad range of taxa, including fish (Ward, 2009). Meanwhile, for the nearest neighbor distance, it was calculated with a minimum distance of 16.78% and maximum distance of 27.08% with a mean value of 21.72% (Table 4). The similar results of having low intra-specific distance compared to the nearest neighbour shown in a study performed on marine molluscs (Sun *et al.*, 2016).

Table 4. DNA barcode gap between ornamental fish specimens from selected pet stores in Seremban, Malaysia**.**

Species	Mean Intra-Sp	Max Intra-Sp	Nearest Species	Distance to (NN)
Betta splendens	0.16	0.16	*Balantiocheilos melanopterus*	27.08
Balantiocheilos melanopterus	0	0	*Carassius auratus*	16.78
Carassius auratus	0	0	*Balantiocheilos melanopterus*	16.78
Puntigrus tetrazona	N/A	0	*Balantiocheilos melanopterus*	22.76
Xiphophorus helleri	N/A	0	*Carassius auratus*	25.19

Note: N/A is not available

The result shows no intra-specific barcode gap value for two species namely *Puntigrus tetrazona* and *Xiphophorus helleri* due to singleton. *Betta splendens* and *Balantiocheilos melanopterus* exhibited the highest distance to nearest neighbour (NN) which is 27.08%. *Betta splendens* belongs to the family Osphronemidae and of order Perciformes meanwhile *Balantiocheilos melanopterus* belongs to the family Cyprinidae and of order Cypriniformes. Both species are from different family and this finding supported their differences in macrohabitat and morphology where *Betta splendens* has streamlined body shape with layered colours which live in thickly overgrown ponds and slowly flowing waters, while *Balantiocheilos melanopterus* has elongated torpedo-shaped body and inhabit in midwater levels (Rainboth, 1996). The same situation happened to a study conducted by Marshita *et al.* (n.d.) where there were differences in macrohabitat and also morphology. Conversely, *Balantiocheilos melanopterus* and *Carassius auratus* showed the lowest distance to nearest neighbor with 16.78%. This is suspected as both species were from the same family, Cyprinidae and inhabit in the same habitat which is in stagnant waters of rivers, lakes, and ponds (Riede, 2004; Man *et al*., 1981; Etnier & Starness, 1993).

Phylogenetic relationship between ornamental fishes

Eight sequences (5 species) of freshwater ornamental fish from own collections and additional conspecific sequences obtained from the GenBank act as replicates, to complement the five species identified (Table 5).

Table 5. List of borrowed sequences from GenBank.

Species	GenBank Accession	Size(bp)	Origin
Puntigrus tetrazona	JF915650	663	Singapore
	JQ667574	670	India
	JQ667572	670	India
Balantiocheilos melanopterus	KU568765	665	South Africa
	KU568764	665	South Africa
Carrasius auratus	EU524448	654	Canada
	KJ552976	653	Greece
Betta splendens	GQ911915	668	Thailand
	GQ911923	668	Thailand
Xiphophorus helleri	LC153769	652	Japan
	HQ219147	656	India
	KJ554749	665	Germany

The phylogenetic tree was constructed by using two different statistical methods of Neighbor-Joining (Figure 2) and maximum likelihood (Figure 3). The bootstrap confidence of 1000 bootstrap replications was implemented through MEGA 7.0 to evaluate highest likelihood of a tree.

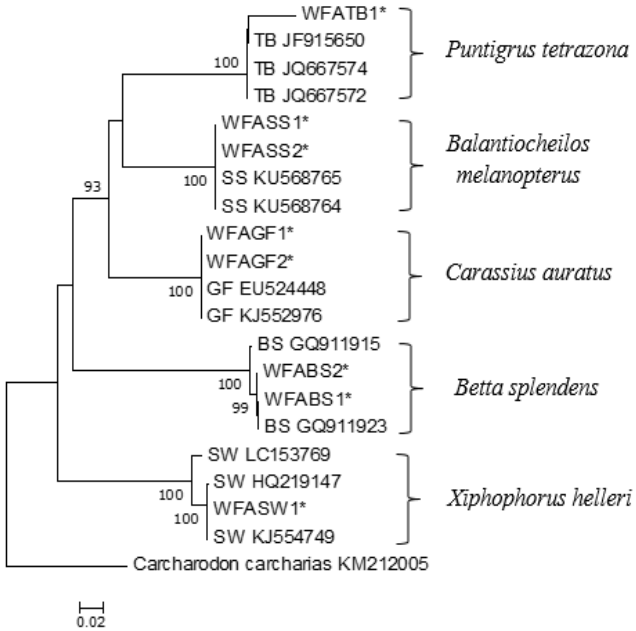

Figure 2. Neighbour-Joining (NJ) tree showing relationship among eight sequences of freshwater ornamental fish (own collections marked with *) with reference dataset (obtained from GenBank). Each node represented with bootstrap value (%) after 1000 replications.

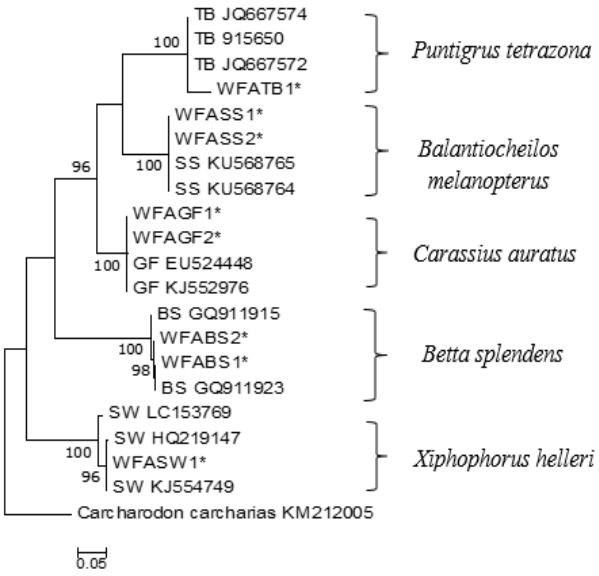

Figure 3. Maximum-likelihood (ML) tree showing relationship among eight sequences of freshwater ornamental fish (own collections marked with *) with reference dataset (obtained from GenBank). Each node represented with bootstrap value (%) after 1000 replications.

The assemblage of own specimens with additional sequences was to aid in confirmation of species assignment, especially the singletons. One outgroup sequence, *Carcharodon carcharias* (KM212005) were included in this analysis to serve as a reference group when determining the evolutionary relationships of the ornamental fishes and specifically allows for the phylogeny to be rooted. Generally, the NJ and ML analysis showed the *COI* sequences for each five species were clearly discriminated from each other. All the specimens from different genus was found with high bootstrap value (n>90%) through Neighbour-Joining (NJ) and Maximum-likelihood (ML) analysis. These result present study by Bhattacharjee *et al*. (2012), intraspecies individuals showed similarity ranged from 97% to 100% clustered cohesively with each other and it is different when it comes to interspecies.

In the NJ and ML tree, the first clade consisted of all species from the same family. The Cyprinidae is monophyletic. Species from Cyprinidae family were grouped together which consisted of *Carassius auratus, Balantiocheilos melanopterus* and *Puntigrus tetrazona* with bootstrap value of 93%. Sister clade was found between *Puntigrus tetrazona* and *Balantiocheilos melanopterus* with mean sequence divergence of 22.76%. This close genetic relationship between *Puntigrus tetrazona* and *Balantiocheilos melanopterus* might be in term of similar morphological characteristics of having similar colour pattern (silver to brownish) all over their body (Kottelat, 2013). The *Puntigrus tetrazona* (WFATB1*) had encountered a gap in pairwise distance (Appendix A) among additional conspecific individuals with divergence of 4% shown through the deeper branch even all specimens were clustered together with strong bootstrap value (100%). Unlike the reference sequences from Singapore (JF915650) and India (JQ667574, JQ667572) that was found with zero gap. This finding came out with the possibility of the fish being traded. The justification is supported with a study by Zhang (2011) said that it might be because of the geographic isolation. The remaining species, *Betta splendens* and *Xiphophorus helleri* displayed as an independent clade. It was due to different genes applied (Quyen *et al*., 2015).

CONCLUSION

In conclusion, DNA barcoding was found to be an effective tool and accurate method in genetic identification. Results showed high similarity in identity when compared to the standard references in BLAST and BOLD database. This indicated that DNA barcoding has high efficiency in species identification. The intra and interspecific genetic divergence is less than 2% which marked a little change in genetic diversity. The current study constructed phylogeny of economic and ecological important freshwater ornamental fish. This data can be accessed through BOLD system which is available to anyone and could provide the sources for the study of biodiversity and management of fisheries resources in Seremban, Negeri Sembilan.

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APPENDIX

Appendix A: Pairwise genetic distance among eight sequences of freshwater ornamental fish (own collections marked with *) with reference dataset (obtained from GenBank).

WFATB1*																			
TB JQ667572	0.040																		
TB_JQ667574	0.040	0.000																	
TB_915650	0.040	0.000	0.000																
WFASS1*	0.228	0.186	0.186	0.186															
WFASS2*	0.228	0.186	0.186	0.186	0.000														
SS_KU568765	0.228	0.186	0.186	0.186	0.000	0.000													
SS KU568764	0.228	0.186	0.186	0.186	0.000	0.000	0.000												
WFAGF1*	0.244	0.199	0.199	0.199	0.168	0.168	0.168	0.168											
WFAGF2*	0.244	0.199	0.199	0.199	0.168	0.168	0.168	0.168	0.000										
GF EU524448	0.244	0.199	0.199	0.199	0.168	0.168	0.168	0.168	0.000	0.000									
GF_KJ552976	0.244	0.199	0.199	0.199	0.168	0.168	0.168	0.168	0.000	0.000	0.000								
WFASW1*	0.336	0.288	0.288	0.288	0.280	0.280	0.280	0.280	0.251	0.251	0.251	0.251							
SW KJ554749	0.336	0.288	0.288	0.288	0.280	0.280	0.280	0.280	0.251	0.251	0.251	0.251	0.000						
SW_HQ219147	0.334	0.288	0.288	0.288	0.280	0.280	0.280	0.280	0.251	0.251	0.251	0.251	0.002	0.002					
SW_LC153769	0.331	0.283	0.283	0.283	0.280	0.280	0.280	0.280	0.237	0.237	0.237	0.237	0.022	0.022	0.024				
WFABS1*	0.318	0.293	0.293	0.293	0.274	0.274	0.274	0.274	0.275	0.275	0.275	0.275	0.305	0.305	0.303	0.318			
WFABS2*	0.316	0.291	0.291	0.291	0.272	0.272	0.272	0.272	0.273	0.273	0.273	0.273	0.303	0.303	0.301	0.316	0.002		
BS GQ911923	0.318	0.293	0.293	0.293	0.274	0.274	0.274	0.274	0.275	0.275	0.275	0.275	0.305	0.305	0.303	0.318	0.000	0.002	
BS_GQ911915	0.308	0.283	0.283	0.283	0.274	0.274	0.274	0.274	0.270	0.270	0.270	0.270	0.295	0.295	0.293	0.308	0.008	0.006	0.008