Pertanika J. Trop. Agric. Sc. 41 (3): 1341 - 1354 (2018)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Phylogenetic and Expression of Atp-Binding Cassette Transporter Genes in *Rasbora sarawakensis*

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ABSTRACT

The ATP-Binding Cassette transporters (ABC transporters) function in various physiological activity, allowing vertebrate to thrive even in polluted environment. The objective of this study is to discover ABC genes in *Rasbora sarawakensis*, a species endemic to Borneo and to understand the respective genes regulation. In this research, nine gene partial transcripts were isolated via RT-PCR and cloning approaches. Our study showed that most gene transcripts identified share high identities with conserved motif distributions across family. Further phylogenetic analysis revealed a clear divergence into three major functional clades (A2, E1, F1; D2; B4, B8, C2, G2). Expression profiles in six tissues (i.e., brain, eye, gill, intestine, muscle, and skin) revealed divergence that shed light on tissue-specific gene functional specialization, with highlight on B4, B8, and E1 which are expressed in all six organs. The brain and eye were also found to express all ABC genes selected in this study. In conclusion, nucleotide profiles of these genes are comparable to the phylogenetic analysis and expression patterns across family. This study implies that an alternative vertebrate model organism can possibly complement the current zebrafish researches.

Keywords: ABC transporters, expression profiles, isolation, phylogeny, *Rasbora sarawakensis*

ARTICLE INFO

Article history:
Received: 07 December 2017
Accepted: 26 January 2018
Published: 29 August 2018

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ISSN: 1511-3701
e-ISSN: 2231-8542

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INTRODUCTION

The ecosystem present in the aquatic environment greatly influences the overall health of the habitat. Various pollutants may be present naturally or introduced into the aquatic habitat by any means, causing significant impact on the aquatic biota (Ferreira, Costa, & Reis-Henriques, 2014; Liu, Li, & Liu, 2013). A handful of ATP-Binding Cassette (ABC) transporters can function to convert these polluting chemicals into their more execrable forms via biotransformation enzymatic reactions and detoxification pathways (Ferreira et al., 2014; Liu et al., 2013).

The ABC transporter superfamily is one of the most expanded family groups of the transmembrane proteins. Initially, this family was identified and characterized based on their functions in multidrug resistance (MDR) (Aryal, Laurent, & Geisler, 2015; Ferreira et al., 2014). The translocations of substances across membranes in biological systems of the host, especially the eukaryotes, are regulated by most members of the ABC proteins equipped with full transporters (Dermauw & Van Leeuwen, 2014; Ferreira et al., 2014).

To date, there are six subfamilies from ABCA to ABCH, that branch out from the ABC superfamily, each representing distinctive homology characteristics related to various functions and disorders (Andersen et al., 2015; Guo et al., 2015; Park et al., 2016). Of all members of the superfamily, only *ABCH1* gene is found to be absent in mammals (Popovic, Zaja, Loncar, & Smital, 2010). *ABCH1* gene had been discovered only in mold, insects and fish but not in other vertebrates. Subfamilies ABCA, ABCB, and ABCC are the groups containing full transporters and half transporters whereas other subfamilies (ABCD, ABCE/ABCF, and ABCG/ABCH) consist of only half transporters.

Thus, there is also a strong need to unravel the potential of using these gene biomarkers in selected endogenous organisms like the Sarawak rasbora for domestic environmental studies (Fedorenkova, Vonk, Breure, Hendriks, & Leuven, 2013; Luckenbach, Fischer, & Sturm, 2014), emulating *Rasbora caverii* as agrochemical model in Sri Lanka (Wijeyaratne & Pathiratne, 2006) and ABC gene discoveries in common carp (Liu et al., 2016). Rasbora genus represents one of the largest family members of the Cyprinidae with eighty-seven species recorded so far (Eschmeyer, 2015). Hence, this research focused on the isolation of nine selected ABC transcripts from *R. sarawakensis* and further followed by the transcript expression verification in various tissues and organs. By determining the presence and expression of these genes, this information can be fed into further studies involving functional characterization of these vital genes involved in ecotoxicology pathways in this fish species in the future.

MATERIALS AND METHODS

Total RNA Extraction

Sampling of *R. sarawakensis* was conducted at the Matang Wildlife Park under the permit NCCD.940 47(Jld1 3) -178 by the Sarawak Forestry Department. Live specimens

caught were then maintained at 26°C under 12-h light and 12-h darkness photoperiod under the Animal Ethics Committee of Universiti Malaysia Sarawak (UNIMAS/TNC(PI)-04.01/06-09 (17). The whole fish body was euthanized on ice and transferred to 1.5 mL microcentrifuge tube for RNA extraction using TRI reagents according to manufacturer's protocol (Sigma-Aldaich, USA). The aqueous phase was transferred to a new tube and total RNA was precipitated with isopropanol at room temperature. The pellet was subsequently dissolved in 20 µL nuclease free water and was stored at -80°C. A total of three biological replicates were used in this study.

Primer Design

In order to identify ABC superfamily genes sequence, FASTA sequences of each selected fish species were retrieved. The Clustal Omega interface website was used to conduct multiple sequence alignment for the ABC gene sequences of the six freshwater fish (Sievers et al., 2011). Based on the results obtained, suitable forward and reverse primers of 20-25 base pairs were selected on the regions of conserved domains. Primer3 software was used to assist in locating the right primer pair (Untergassar et al., 2012). The primers were screened for melting temperature, presence or absence of secondary structure, self-complementary as well as GC content using OligoCalc tool (Kibbe, 2007). The selected primer orders were sent to First BASE Laboratories Sdn. Bhd. for primer synthesis (Table 1).

First Strand cDNA Synthesis

EasyScript® Reverse Transcriptase (TransGen, China) was used to synthesize first-strand cDNA strand. Total RNA, primer and RNase-free water were mixed in a sterile PCR tube before incubating the tube at 65°C for 5 min, then on ice for 2 min. Then, other components such as dNTP mix, EasyScript® Reverse Transcriptase and

Table 1
The forward and reverse primers used in PCR amplification of selected ABC genes and β-actin

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
ABCA2	GATGT*D*GATGTGGCCTGTGA*R*AGA	CCAGC*W*GG*Y*TTGTC*K*GCATACTT
ABCB4	GTCCT*D*AATGG*Y*ATGAATCT	CCAGC*R*AT*K*ACATCAGCATT
ABCB8	TTCAGTTA*Y*CCAAC*R*AGACC	CCTCCTTTG*S*TCAG*Y*A*R*TTC
ABCC2	GG*K*AA*Y*CTGGT*R*GTGTTTTT	ACACA*R*CA*R*CTG*Y*CTCTGA
ABCD2	ATGGT*B*GC*Y*GTGCC*Y*ATCAT	CTGCTTCTCTCCAGA*H*A
ABCE1	GCCAA*Y*TCCTTCAA*R*CTGCA	ACAGCAGATGAAGTC*N*GACA
ABCF1	GCTGT*Y*ATCTGGCT*B*AACAACTA	TTGGA*R*ATCTG*R*AT*R*GTGTG
ABCG2	ACCGTCAGCTTCCACAACATC	GA*Y*GGAGAACA*R*GAAG*R*TGAAGA
ABCH1	CATCAGGCTTTTGAGGCTTT	TGATTGGCAGATCCATGTGT
β-actin	GGAGGAGATCTGGCATCACAC	GATCTCCTTCTGCATCCTGTCA

Reverse Transcriptase Buffer were added into the mixture and incubated consecutively according to the manufacturer's protocol.

Gradient RT-PCR

The optimum temperatures of all gene primers and *β-actin* were determined and applied for the following RT-PCR reactions in a T100™ Thermal Cycler (Bio-Rad, USA). RT-PCR was performed in a 20 µL reaction tube by preparing the 4X master mix containing 1X *EasyTaq* Buffer (with Mg^{2+}), 0.2 mM dNTPs, 0.2 µM forward and reverse primers respectively, 2.5 units *EasyTaq* DNA polymerase (TransGen, China), 2.5 ng/µL cDNA and nuclease-free water. The mixtures were subjected to one cycle of pre-denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55-60°C for 30 s and extension at 72°C for 40 s as well as one cycle of final extension at 72°C for 5 min. Three replicates were used for each gene in RT-PCR reactions. Gel extraction was conducted using Wizard® SV Gel and PCR Clean-Up System according to manufacturer's protocol (Promega, USA). Selected gene transcripts were cloned into pGEM-T® Easy vector (Promega, USA) according to manufacturer's protocol before they were subjected to sequencing.

Sequencing and Analysis

Purified PCR products and plasmids were sent to First BASE Laboratories Sdn. Bhd. for sequence determination using the same primers used for RT-PCR amplification (for PCR product sequencing) and T7 promoter primer (for plasmid sequencing). The electropherogram files were analyzed using the Mega7 (Kumar, Stecher, & Tamura, 2016) to trim and obtain consensus sequences through a quality check. Then, the results of the output obtained from direct sequencing were analyzed using basic local alignment search tool (BLAST) from NCBI (Altschul, Gish, Miller, Myers, & Lipman, 1990). Motif distribution was inspected by using MEME tool (Bailey & Elkan, 1994). ORFs were predicted from these gene transcripts using NCBI ORFfinder (Rombel, Sykes, Rayner, & Johnston, 2002), blasted using BLASTp and sequences of motifs were revealed via NCBI CDD (Marchler-Bauer et al., 2016). The amino acid sequences (predicted ORFs) were subjected to Best Protein Model test using Mega7 and Jones-Taylor-Thornton (JTT) model using maximum likelihood was revealed to be the best model. A maximum likelihood phylogenetic tree (Bootstrap method, replications of 500, Jones-Taylor-Thornton (JTT) model) was constructed to compare ABC genes across family members of Cyprinidae using Mega7 (Kumar et al., 2016).

Semi-Quantitative Expression Analysis

First, a total of ten *R. sarawakensis* fishes were euthanized by using Tricaine. Organs such as the brain, eyes, gills, intestine, muscle and skin were isolated. The six organs of *R. sarawakensis* were sliced on petri dish while on ice and then they were transferred into six sterile labeled 1.5 mL microcentrifuge tubes, respectively. The six organ samples were homogenized

with 500 µL of TRI® Reagent according to manufacturer's protocol (Sigma-Aldaich, USA) in respective sterile microcentrifuge tubes by using micropipette tips. These RNAs were extracted and used for cDNA synthesis according to the manufacturer's protocol. The cDNA of six organ samples were kept in -20°C for further expression study. These cDNAs of six organs were subjected to RT-PCR conditions as optimized above, using the ABC gene primers designed previously with three replicates, together with beta-actin gene as positive control to determine the semi-quantitative expression of these genes on agarose gel.

RESULTS AND DISCUSSION

Motif Distribution and Protein Analyses

To isolate ABC transporter genes, total RNAs were extracted from whole body of Sarawak rasbora. A total of nine ABC transporter gene transcripts have been isolated from *R. sarawakensis* with at least one representative member from a total of six subfamilies. Degenerate primers were designed based on conserved motifs that are shared within family members of Cyprinidae (i.e., zebrafish, common carp, and *Sinocyclocheilus* spp.). These transcripts isolated were found to have lengths ranging between 464-1383 bp (Table 2). Besides, they were also analyzed by BLASTn and most of them have identities ranging from 74-94% to that of zebrafish except for *ABCD2*, *ABCF1* and *ABCH1*. The BLASTp results (Table 2) were found to be consistent with that of BLASTn results, most of the gene transcripts isolated from the Sarawak rasbora hit the highest score to that of the zebrafish except for *ABCA2, ABCF1* and *ABCH1*. These ABC gene transcripts of Sarawak rasbora shared high identities in terms of protein to that of the zebrafish with percentage as high as above 95%. The BLASTp identities shared between close family members of Cyprinidae and the Sarawak rasbora are generally higher than that of nucleotides, indicating the strong functional conservation of these genes across the Cyprinidae family despite the presence of nucleotide variations due to speciation.

ORFs were predicted from these gene transcripts *in silico* using NCBI ORF finder, blasted using BLASTp and sequences of motifs were revealed via NCBI CDD (Table 3). These ORFs were found to be protein isoforms that are predicted from all 9 intronless transcripts. The motif distribution and location were found to be high conserved across the ABC family members of Sarawak rasbora except for *ABCH1* (Figure 1). The Walker A and Q-loop were found in all ABC gene transcripts with the exception for *ABCH1*. The other motifs such as ABC Signature, Walker B, D-loop, and Q-loop, were found to be conserved in terms of location and distribution in the gene transcripts they were found in, suggesting their concerted functional roles in detoxification that allows teleost to strive and adapt in polluted environments. The predicted ORFs used in this study was the product of *in silico* translation used to predict structural features of these genes.

Table 2
The summary of BLASTn and BLASTp results of the selected ABC genes

Gene isolated from Sarawak rasbora	Length of transcript isolated	BLASTn highest score potential candidate	Identities (%)	BLASTp highest score potential candidate	Identities (%)
ABCA2 (MG757499)	464 bp	*Danio rerio ABCA2* XM_005165231.4	92	Predicted *S. rhinocerous ABCA2-like* XP_016413565.1	100
*ABCB4** (MG757500)	534 bp	*Danio rerio ABCB4* NM_001114583.2	90	*Danio rerio ABCB4* isoform 2 NP_001108055.2	97
ABCB8 (MG757501)	668 bp	*Danio rerio ABCB8* NM_001017544.1	87	*Danio rerio ABCB8* NP_001017544.1	97
ABCC2 (MG757502)	696 bp	*Danio rerio ABCC2* NM_200589.2	85	*Danio rerio ABCC2* AAI55106.1	95
*ABCD2** (MG757503)	588 bp	Predicted *Sinocyclocheilus grahami ABCD2-like* XP_016107888.1	90	Predicted *Danio rerio ABCD2-like* XP_005174656.2	100
ABCE1 (MG757504)	600 bp	*Danio rerio ABCE1* AAH45882.1	94	*Danio rerio ABCE1* NP_998216.2	100
*ABCF1** (MG757505)	679 bp	Predicted *Sinocyclocheilus anshuiensis ABCF1-like* XP_016345625.1	91	*Oryzias latipes ABCF1* XP_004074386.3	100
*ABCG2** (MG757506)	1383 bp	*Danio rerio ABCG2a* NP_001036240.1	85	*Danio rerio ABCG2* NP_001036240.1	96
*ABCH1**	678 bp	*Cyprinus carpio* genome assembly common carp genome, scaffold: LG12, chromosome: 12 LN590717.1	74	-	-

Table 3
The ORFs of each partial gene fragment predicted by NCBI ORFfinder

Gene	Amino acid length*	Motifs available	Corresponding motif sequences
ABCA2	153 aa	Walker A/P-loop	GVNGAGKT
		Q-loop/lid	YCPQ
ABCB4	177 aa	Walker A/P-loop	GSSGCGKS
		Q-loop/lid	VVSQ
		ABC signature	MSGGQKQRIA
		Walker B	ILLLDE
		D-loop	SALD
		H-loop/switch region	IVVAHRL
ABCB8	221 aa	Walker A/P-loop	GESGGGKST
		Q-loop/lid	FISQ
		ABC signature	LSGGQKQRIA
		Walker B	ILILDE
		D-loop	SALD
		H-loop/switch region	LIIAHRL
ABCC2	231 aa	Walker A/P-loop	GRTGAGKS
		Q-loop/lid	IIPQ
		ABC signature	LSLGQRLLC
ABCD2	194 aa	Walker A/P-loop	GPNGCGKS
		Q-loop/lid	YIPQ
ABCE1	199 aa	Walker A/P-loop	GTNGIGKST
		Q-loop/lid	VKPQ
		ABC signature	LSGGELQRFA
		Walker B	IFMFDE
		D-loop	SYLD
		H-loop/switch region	IVVEHDL
ABCF1	212 aa	Walker A/P-loop	GPNGVGKS
		Q-loop/lid	FFNQ
ABCG2	231 aa	Walker A/P-loop	RATGSGKSS
		Q-loop/lid	YVVQ
		ABC signature	VSGGERKRTN
		Walker B	VLFLDE
		D-loop	TGLD
		H-loop/switch region	ILSIHQP
ABCH1	224 aa	No putative motifs detected	-

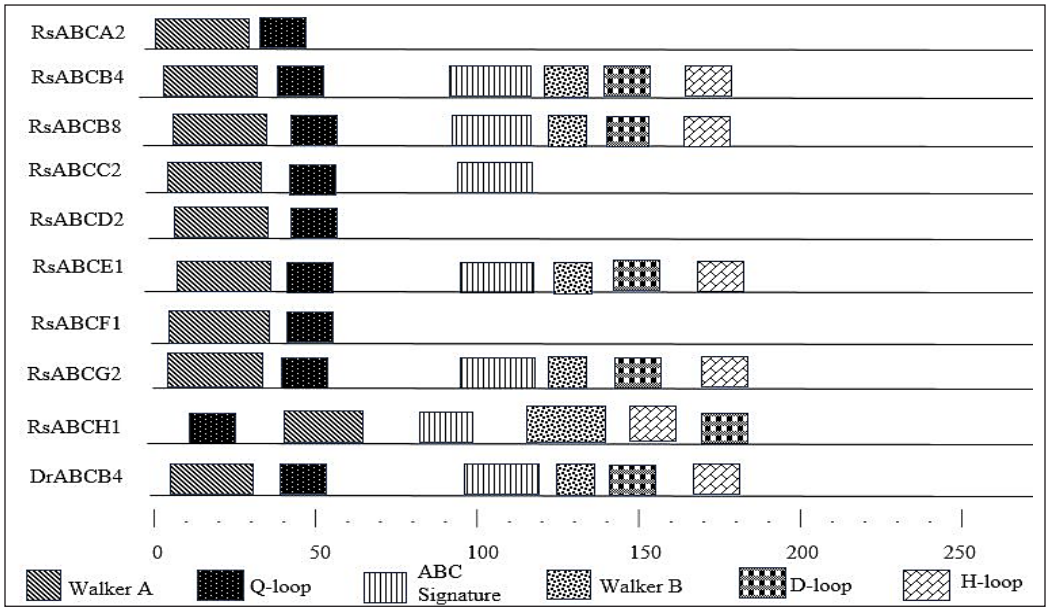

Figure 1. Motif distribution of selected ABC genes in Sarawak rasbora generated by MEME tool (Rs: *R. sarawakensis*; Dr: *Danio rerio*)

More advanced work has to be done in subsequent experiments to isolate the full ORFs using GeneRacer™ kit (Thermo Fisher Scientific, USA) in the future.

Phylogenetic Analysis

The phylogenetic tree constructed using maximum likelihood criterion gave a rather clearer point of clustering. Based on the ABC family tree constructed (Figure 2), it was observed that these ABC family members are clustered into three main clades where *ABCB4*, *ABCB8*, *ABCC2,* and *ABCG2* were grouped into Cluster A, *ABCD2* in its own Cluster B, while *ABCA2*, *ABCE1,* and *ABCF1* were grouped into Cluster C.

The phylogenetic clade where *ABCB4*, *ABCB8*, *ABCC2,* and *ABCG2* resided (Cluster A) has shown bootstrap values ranging up to 99% within clades. This cluster has been previously characterized as the canalicular ABC transporters (Cuperus, Claudel, Gautherot, Halilbasic, & Trauner, 2014; Paulusma et al., 1997; Smit et al., 1993). The canalicular phospholipid flippase (*ABCB4* protein) functions to regulate biliary excretion of phospholipids, synthesizing mixed micelles along with cholesterol as well as bile acids, thus provide protection for the bile duct epithelium against bile acids (Trauner, Fickert, Halilbasic, & Moustafa, 2008). Loss of function of *ABCB8* can result in interruption of iron homeostasis between cytosol and mitochondria in mouse. Besides, this gene, if found defective, can lead to severe cardiac dysfunction in mice (Ichikawa et al., 2012). *ABCC2*, *ABCG2* together with *ABCB1* are canalicular ABC transporters that are responsible for biliary

excretion of xenobiotics (Cuperus et al., 2014). These genes are major parts of the multidrug resistance mechanism which are greatly influenced by drug-drug interactions and these genes can contribute to cholestatic diseases, if found defective (Jäger, 2009).

Cluster B was only resided by *ABCD2* gene transcript of Sarawak rasbora. The *ABCD2* clade interestingly displayed the maximum branching within clade when compared to clade from other clusters. With bootstrap values within clade ranging between 72-99%, the *ABCD2* of Sarawak rasbora seem to branch off further than its counterparts from other fish from the same family.

Cluster C where *ABCA2*, *ABCE1* and *ABCF1* are clustered together has not been previously characterized in depth, unlike Cluster A. Cluster C depicted high bootstrap values up to 100%. This cluster shows a much closer clade relationship with less branching happening within clades in this cluster (Figure 2). The identities of clade members, especially clade for *ABCE1* and *ABCF1* within clades, were found to be higher and they were more evolutionarily conserved in terms of nucleotide and protein sequences compared to members from other clusters. More functional studies have to be done in future to further characterize them into sub-clusters based on their functional roles and tissue specificities.

Expression Analysis

Expression analysis was also done on these ABC gene transcripts (Figure 3) to study their expression patterns as well as levels of expression. Another internal reverse primer was designed to amplify a shorter fragment of *ABCG2* gene transcript for expression purposes. There were some transcripts of Sarawak rasbora that were found to express in all six organs selected (i.e., brain, eye, gill, intestine, muscle, and skin), namely *ABCB4*, *ABCB8,* and *ABCE1*. The *ABCA2*, *ABCC2*, *ABCF1,* and *ACBG2* however were discovered to have expression in five organs except the skin. It is also interesting to observe that the brain and eye organs were found to express all eight gene transcripts of Sarawak rasbora. The expression of *ABCD2* was rather specific, it was only being expressed in the brain and eye.

Currently, the expression profiles of ABC genes (although not all) are only available for zebrafish and common carp within the Cyprinidae family. From the comparisons of expression data between the two species and Sarawak rasbora, a few highlights had been focused on. The intestine organ was found to have high expression of *ABCA2* in Sarawak rasbora and common carp, *ABCG2* in zebrafish and Sarawak rasbora as well as *ABCB4*, *ABCC2* and *ABCE1* in all 3 species (Fischer et al., 2013; Liu et al., 2016; Long, Li, Zhong, Wang, & Cui, 2011; ZFIN, 2018). The expression of ABC genes in gill was seen for *ABCE1* in Sarawak rasbora and common carp, *ABCG2* in zebrafish and Sarawak rasbora as well as *ABCC2* for all 3 species (Kobayashi et al., 2008; Liu et al., 2016; Long et al., 2011). The brain expression was found common in terms of *ABCB4*, *ABCC2*, *ABCE1,* and *ABCF1*

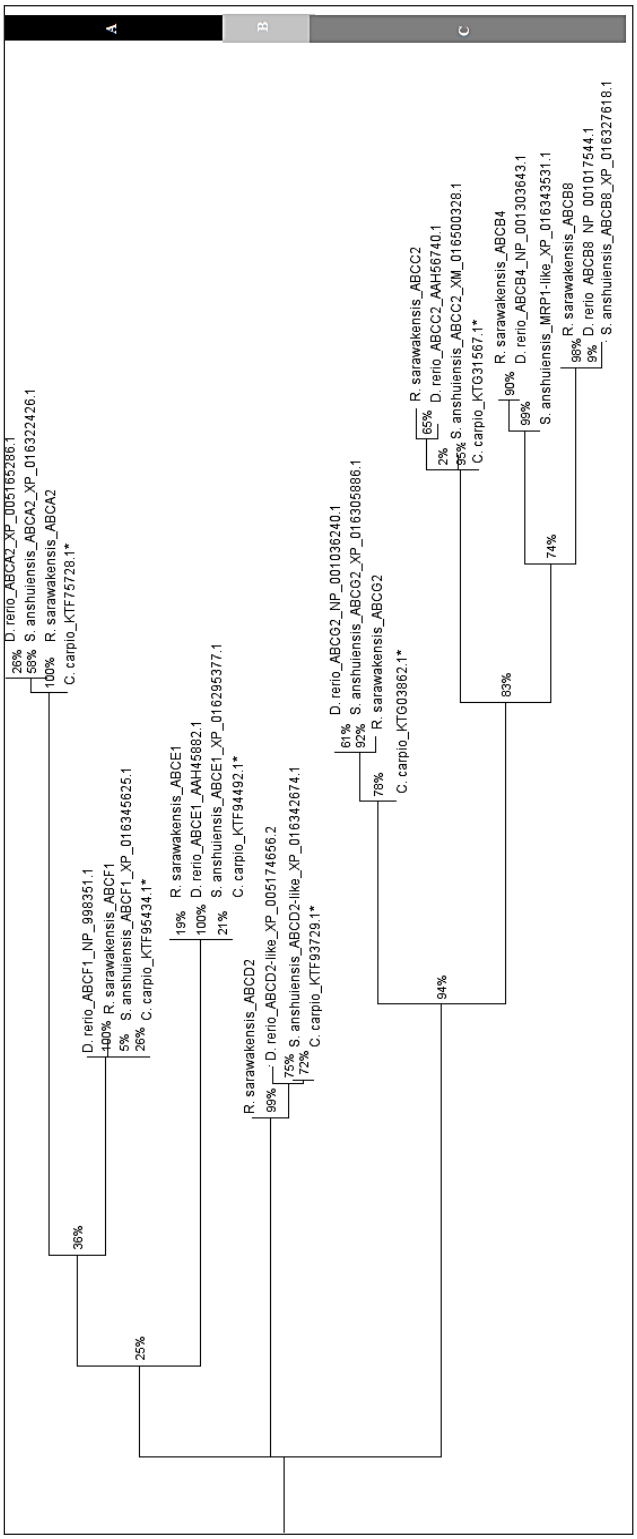

Figure 2. Phylogenetic maximum likelihood tree constructed using bootstrap with 500 replications, showing Cluster A, B, and C respectively. ("*" indicates hypothetical protein)

between Sarawak rasbora and common carp (Liu et al., 2016). The absence of *ABCD2* in both gill and intestine was found similar between Sarawak rasbora and common carp (Liu et al., 2016). These findings suggest that most of the conserved physiological roles of ABC genes take place in these three organs: intestine, gill and brain, across the Cyprinidae family.

From the expression patterns of the four gene transcripts of Sarawak rasbora (*ABCB4*, *ABCB8*, *ABCC2,* and *ABCG2*) from Cluster A, it was observed that most of them are expressed in almost all 6 organs except for *ABCC2,* and *ABCG2*. The *ABCG2* was not expressed in the skin of the Sarawak rasbora. This may be due to the functional compensation mechanism

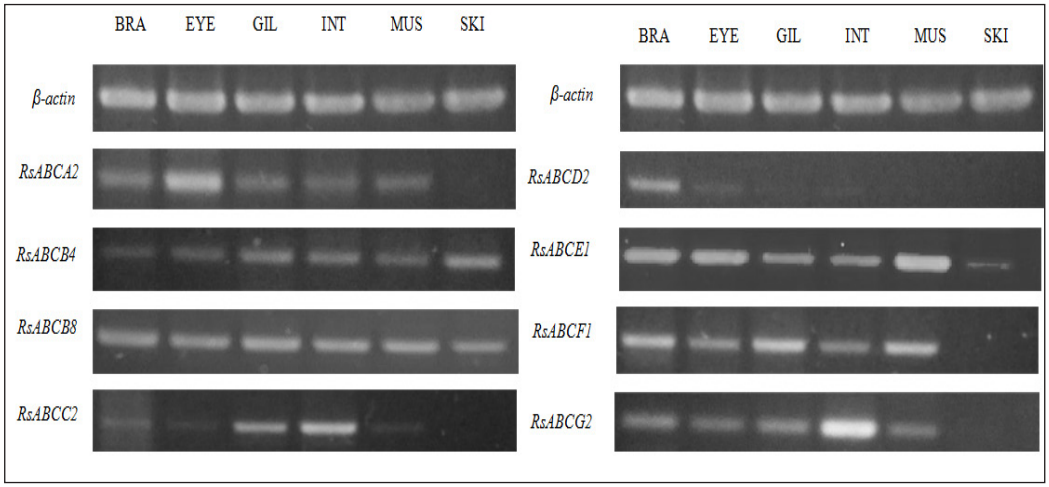

Figure 3. Expression patterns of ABC gene transcripts of Sarawak rasbora with *beta-actin* as positive control. BRA: brain, EYE: eye, GIL: gill, INT: intestine, MUS: muscle, SKI: skin.

where they share substrates as well as sites of expression and this is also why *ABCG2* knockdown mice did not suffer from severe phenotypes (Cuperus et al., 2014). The high expression pattern of *ABCC2* in the gill and intestine may explain its possible role in the detoxification mechanism as proven in the case of Nile tilapia, where expression of *ABCC2* was elevated up to 16-fold following benzo(a)pyrene exposure (Costa, Reis-Henriques, Castro, & Ferreira, 2012).

Considering the expression patterns of gene members of Sarawak rasbora from Cluster C, it can be observed that all gene members were expressed in the brain organ and the expression levels were relatively high. This may provide insights into the involvement of these ABC genes in the mechanisms of transport that are specifically localized in the brain.

CONCLUSION

In this study, a total of nine ABC transporter genes were identified in the Sarawak rasbora genome. The phylogenetic analysis had provided us more understanding towards the ABC gene family in the evolution of teleosts. The highly conserved motif distribution

across ABC gene family members suggests that they are the potential targets for the study of interactions involved in the mechanism of ecotoxicology especially in the teleosts, thus providing more detailed comprehension on how they can strive to live and adapt in polluted environment.

The expression profiles of most of the gene transcripts from the Sarawak rasbora correlate with the phylogenetic clustering where some similar characteristics were observed. This study serves as a preliminary exploration into evolution (i.e. divergence and convergence) of this gene family in the Sarawak rasbora. The functional significance and structural diversity of this gene family in teleost are yet to be explored in detail. In future, further characterization of the complete gene transcripts thoroughly will be performed via functional studies to verify their tissue-specific functions.

ACKNOWLEDGMENT

This work was fully funded by The Ministry of Higher Education, Malaysia through the Fundamental Research Grant Scheme FRGS/SG05(03)/1148/2014(15).

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