Int Aquat Res (2023) 15:163–179
https://doi.org/10.22034/IAR.2023.1980719.1402

ORIGINAL RESEARCH

Antibacterial screening of epidermal mucus protein extract of freshwater Bornean spotted barb *Barbodes sealei*

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Received: 27 February 2023 / Accepted: 27 May 2023 / Published online: 10 June 2023

Abstract The epidermal mucus of fish serves as the first line of defence against the microbe-rich aquatic environment, containing various innate immune components, including antimicrobial proteins. However, information regarding the antibacterial properties of skin mucus of Bornean native fish is scarce. This study aims to enhance the understanding of the epidermal mucus of *Barbodes sealei,* a Bornean endemic freshwater fish species. Pooled mucus samples were extracted using saline (aqueous extract) and acetic acid (acidic extract). The extracts were purified and concentrated through ammonium sulfate precipitation. This study presents the antibacterial screening of these mucus extracts against 16 selected bacterial strains. The results revealed that among the bacterial strains tested, only *Salmonella braenderup* ATCC BAA 664 showed sensitivity to the acidic extracts, while none of the aqueous extracts exhibited any antibacterial activity. The findings suggest that higher protein contents in the extracts did not necessarily correlate with better antibacterial activities. To identify the major proteins present in the active extracts and determine the antibacterial proteins, a qualitative bioanalysis was conducted using high-throughput Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS). Four antibacterial proteins, namely Histone H2A, Histone H2B, Histone H4, and Heat shock protein 70, were identified based on comparison with existing literature. Further isolation and characterisation of the active components, particularly the antimicrobial proteins, are warranted to gain deeper insight into their role in fish immunity. This study establishes the antibacterial potential of epidermal mucus from *B. sealei* and proposes it as a non-invasive source for the isolation of new biologically active compounds, such as antimicrobial proteins and peptides.

Keywords Antibacterial activities . *Barbodes sealei* . Borneo . Fish epidermal mucus . Histone H2A . Histone H2B . Histone H4

Introduction

Throughout human history, fish has been recognized as a significant resource, serving not only as a nutritious food source rich in protein and lipids but also as valuable trade commodities, including ornaments and medicines (Tilami and Sampels 2017). To date, there have been over 33,000 described and reported fish species (Froese and Pauly 2023), with more than 40% of them thriving in freshwater habitats (Lundberg et al. 2000; Tedesco et al. 2017). This is remarkable considering that freshwater ecosystems cover only a small portion of the Earth's surface, approximately 0.8%, and make up less than 0.02% of global water (Dudgeon et al. 2006). In Borneo, there are 23 families of freshwater fishes that are confined to freshwater systems and exhibit little tolerance to saltwater (Berra 2007). Among these families, Cyprinidae, which

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includes barbs, carps, and minnows, is the most dominant group in freshwater habitats, comprising over two-thirds of the total freshwater fauna (Sulaiman and Mayden 2012). One notable endemic species found in Borneo is *Barbodes sealei*, the Bornean spotted barb locally referred to as "Turungau" (Inger and Chin 1962; Froese and Pauly 2023). This species is typically found in clear or slightly murky, unpolluted forest streams with sandy or gravelly riverbeds (Inger and Chin 1962). It can be identified by a row of equally spaced dark blotches along its flank.

Unlike terrestrial animals, fish spend their entire life in an aquatic environment. Most aquatic habitats are teeming with saprophytic, pathogenic, and non-pathogenic microbes, such as bacteria, viruses, and fungi (Magnadottir 2010). As fish have continuous contact with their aquatic surroundings and rely on gill-breathing, they are more susceptible to a wide range of diseases. The success of an infection largely depends on the ability of pathogens to adhere to the mucosal surfaces of fish (Magariños et al. 1995; Benhamed et al. 2014). Thus, fish rely heavily on their complex and fast-acting innate immune mechanisms to combat the constant threats to their health (Ellis 2001; Arellano et al. 2004). In general, the innate immune system of fish comprises various organs, including scales, gills, gut, and epidermis, along with the mucus secreted by epithelial cells (Esteban 2012). One of the most crucial components of the fish's innate immune response is the mucous layer that covers their body surface. Mucus is a viscous colloid gel that forms an adherent layer cover, serving as the primary interface between the environment and the interior milieu of the fish. It is continuously secreted and sloughed off as fish encounter, monitor, and regulate the vast microflora present in the aquatic environment, thereby preventing the adherence of pathogens to the underlying tissues (Esteban and Cerezuela 2015). Beyond its role as a physical barrier in the innate defence system, fish skin mucus actively prevents microbial infections and is considered a crucial immunological factor. Epidermal mucus in fish primarily consists of approximately 95% water and glycoproteins, along with various other substances (Bansil and Turner 2006). It contains a wide range of innate immune components, including lysozymes, calmodulin, complement, proteolytic enzymes, lectins, C-reactive proteins, immunoglobulins, as well as antimicrobial peptides and proteins (Shephard 1994; Magnadóttir 2006; Alvarez-Pellitero 2008; Esteban 2012).

Presently, a growing body of research on the antimicrobial function of fish skin mucus suggests that it plays a role in preventing the invasion of parasites, bacteria, and fungi (Hellio et al. 2002; Subramanian et al. 2008b; Lee et al. 2020; Tiralongo et al. 2020). Most of the studies have focused on commercially important farmed or marine species such as Atlantic cod (*Gadus morhua*) (Magnadóttir et al. 2018), Atlantic salmon (*Salmo salar*) (Provan et al. 2013), discus fish (*Symphysodon aequifasciata*) (Chong et al. 2006), European seabass (*Dicentrarchus labrax*) (Cordero et al. 2015), and gilthead seabream (*Sparus aurata*) (Cordero et al. 2016). However, the antimicrobial potential of mucus from freshwater fish, particularly the native species in Borneo, remains unexplored. Therefore, knowledge of skin mucus of Bornean fish species and their innate defence mechanisms can be crucial to overcoming the challenge of combating multidrug-resistant pathogens. This study explores the potential antimicrobial properties of the epidermal mucus of a Bornean endemic freshwater fish species, *Barbodes sealei,* by screening antibacterial activities and sequencing peptides of the active mucus extracts using LC-MS/MS.

Materials and methods

Preparation of the fish mucus extracts

In the study, all procedures were conducted with the approval of the UNIMAS Animal Ethics Committee (UNIMAS/AEC/R/F07/020). *Barbodes sealei* were collected from the upstream river near Melaban Village in Kota Samarahan District (1.5025 °N, 110.4080 °E) using homemade minnow traps with commercial fish feed pellets as attractants. After a seven-day acclimatisation period, thirty healthy fish were selected for the collection of epidermal mucus using methods modified from Subramanian and co-workers (2008b). Prior to the mucus collection, the fish were starved for 24 hours and rinsed with sterile distilled water. They were then placed in a sterile zip-locked polyethylene bag containing 30 ml of physiological saline solution (0.85 % NaCl) and massaged gently for 10 to 15 min to slough off the mucus. The fish were returned to a recovery tank afterwards, while the collected mucus samples were immediately pooled and stored at -4 °C.

The aqueous extract was prepared according to the methods modified from Loganathan and colleagues

(2011). A total of 50 ml of the pooled mucus samples was vortex-mixed to ensure homogeneity. The mixture was then subjected to centrifugation (15 min, 5200 × g, 25 ℃) and filtration (Surfactant-free Cellulose Acetate (SFCA) syringe filter, 0.22 µm pore size, 28 mm diameter), and the resulting supernatants (mucus extract in saline) were collected and stored at -4 ℃ for further use within one week.

For the acidic extract, the method used was modified based on Al-Rasheed and co-workers (2018). A total of 400 µl of 100% (v/v) glacial acetic acid was vortex-mixed with 50 ml of pooled mucus to produce a fully equilibrated mixture comprising one mucus part and four moderately 1% (v/v) acetic acid parts. To inhibit proteolytic enzyme activity (Conlon 2007), the mixture was subjected to a 3-minute boiling water bath, and then cooled on ice. Afterwards, the mixture underwent centrifugation (35 min, 25000 × g, 4 ℃) and filtration (Surfactant-free Cellulose Acetate) syringe filter, 0.22 µm pore size, 28 mm diameter), and the resulting supernatants (mucus extract in moderately 0.8% acetic acid) were stored at -4 ℃ for further use within one week. Negative controls for both extracts were included in the study. The preparation followed the same extraction procedure using either the physiological saline or acetic acid solvents, but without any mucus.

Protein purification by ammonium sulphate

Proteins from both mucus extracts were purified and concentrated using ammonium sulfate precipitation. Solid ammonium sulphate was added to the extracts until reaching 90 % saturation and left overnight at 4 °C to allow complete precipitation of the proteins. After centrifugation (20 min, 15000 × g, 4 ℃), the protein pellets were resuspended in their respective solvents (saline for the aqueous extract and 0.8 % acetic acid for the acidic extract) and dialysed against the same solvent using dialysis tubing with a cellulose membrane (Sigma-Aldrich; MWCO 14 kDa) to remove salt. The resulting product was subjected to further analysis. Protein concentration was determined using the Bradford protein assay (Bradford 1976) with bovine serum albumin as the quantification standard.

Screening of antibacterial activities

The extracts were tested for their *in vitro* antibacterial activities against three Gram-positive bacterial strains (*Bacillus cereus* ATCC 33019, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923) and thirteen Gram-negative bacterial strains (*Aeromonas hydrophila*, *Escherichia coli* O157:H7, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella braenderup* ATCC BAA 664, *Salmonella enteritidis* ATCC 13036, *Salmonella typhi* ATCC 14028, *Salmonella typhimurium*, *Shigella boydii* ATCC 9207, *Shigella flexneri* ATCC 12022, *Shigella sonnei* ATCC 25931, *Vibrio cholerae*, and *Yersinia enterocolitica*). All sixteen bacterial species were cultured in Luria-Bertani (LB) broth and maintained on Muller Hinton (MH) slant agar. The LB-glycerol stock cultures were stored at -20 ℃.

The antibacterial screening of mucus extracts was conducted using Kirby-Bauer disc diffusion method (Bauer et al. 1966). Briefly, a standardised inoculum (100 µl) with an OD_{600} of 0.1 was spread evenly on a MH agar plate using a cell spreader. Sterilized 6 mm paper discs (Brand: Whatman; Grade 1, 11 µm pore size) were then impregnated with the tested mucus extracts (20 µl) and placed evenly on agar surface. After one-hour pre-diffusion at 4 °C, the agar plates were incubated at 37 °C for 16-20 hours. The experiments were performed in triplicates. The clear inhibition zones around the discs were measured in terms of Inhibition Zone Diameter (IZD) and recorded in mm up to one decimal place. The data were presented as mean ± standard deviation. Negative controls, which consisted of saline for the aqueous extract and 0.8% acetic acid for the acidic extract, were tested on the same plate to account for the influence of the solvent used in mucus extracts, while an antibiotic agent named Ciprofloxacin (Brand: Oxoid; 5 µg; Cat no. CT0425B) was used as the positive control. One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to determine significant variation in the antibacterial strength of mucus extracts or positive control against different bacterial strains. An independent Student t-test was conducted to determine significant differences between the mean IZDs of the mucus extracts and their respective negative controls in cases where both exhibited bacterial inhibition against the same strain. Statistical significance was considered at a p-value ≤ 0.05. All statistical analyses were performed using IBM SPSS Statistic 27 version. 3.3.

The minimum inhibitory concentration (MIC) of the active mucus extract was determined using a broth

microdilution susceptibility test. In this test, a two-fold dilution of the mucus extract (100 µl) was prepared on a 96-well (12 × 9) microplate. Each well was then inoculated with a standardised inoculum (OD_{600} = 0.1). After thorough mixing, the microplate was incubated at 37 °C for 16-20 hours. The MIC represents the lowest concentration of the mucus extract that inhibited the bacterial growth. All assays were conducted in triplicates. The data for the antibacterial activity before ammonium sulphate precipitation is not included here, as the extract concentrations were low and did not exhibit any biologically significant activity after testing. The subsequent tests and analysis focused on the post-precipitation extracts with higher concentrations to obtain robust and meaningful findings, in line with the objectives of the study.

Protein characterisation of active mucus extracts

The active mucus extract based on the disk diffusion test was analysed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) buffer system (BioRad Mini-PROTEAN® Tetra Cell; Cat no. 165-8000) with a 12 % (w/v) resolving gel and a 4 % (w/v) stacking gel (Laemmli 1970) and visualised by Coomassie Brilliant Blue R-250 (BioRad) staining. To prepare the SDS-PAGE sample, 10 µl of the active mucus extract was mixed with 10 µl of sample buffer. Then, 20 µl of this mixture was carefully loaded into the gel. A chromatein pre-stained protein ladder (Vivantis Technology, Malaysia; Cat no. PR0602) was used as a standard to determine the molecular weight of the distinct protein bands. The five most predominant protein bands observed at 24 kDa, 40 kDa, 50 kDa, 56 kDa and 66 kDa respectively, were excised from the destained gel and subjected to peptide sequencing using LC-MS/MS (Proteomic International Pty Ltd, Broadway, Nedlands, Western Australia).

Results

The mucus samples were divided into two parts for the aqueous and acidic extraction. Prior to the extraction, the protein concentration was determined to be 0.212 ± 0.016 mg/ml, with a corresponding protein yield of 10.609 ± 0.797 mg. The aqueous extraction slightly decreased the protein concentration to 0.207 ± 0.197 mg/ml, with a corresponding protein yield of 10.387 ± 0.983 mg. Subsequent precipitation with $(NH_4)_2SO_4$ measured the protein concentration at 2.473 ± 0.301 mg/ml and a recovery rate of 23.28 ± 1.60%. Conversely, the acidic extraction significantly decreased the protein concentration to 0.090 ± 0.013 mg/ml, resulting in a total protein yield of 4.495 ± 0.656 mg. Subsequent precipitation with $(NH_4)_2SO_4$ measured the protein concentration at 2.414 ± 0.300 mg/ml and a recovery rate of 22.85 ± 3.52%. Despite the low recovery rates for both extracts, the resulting mucus extracts were 10 times more concentrated than prior to the extraction (see Table 1).

During the preliminary antimicrobial screening, the aqueous extracts of *B. sealei* proved inactive against all the tested strains (data not shown). In contrast, the acidic extracts exhibited antimicrobial activity against most of the tested bacterial strains, including both Gram-positive and Gram-negative species. The only exceptions were *Listeria monocytogenes* ATCC 7644 and *Yersinia enterocolitica*. It is noteworthy that the negative controls of the acidic extracts also displayed similar activity to that of the acidic extracts from *B. sealei*. To confirm that the observed antibacterial activity was not influenced by the solvent used, the IZD values of the acidic extract and its negative controls were subjected to an Independent Two-Sample t-test,

Table 1 Protein concentrations and recovery of *Barbodes sealei* epidermal mucus via aqueous and acidic extractions and upon $(NH_4)_2SO_4$ precipitation.

Extraction method	Volume (ml)	Concentration (mg/ml)	Total Protein (mg)	*Recovery %
Aqueous Extract				
Before Extraction	50	0.212 ± 0.016	10.609 ± 0.797	100 ± 0.00
After Extraction	50	0.207 ± 0.197	10.387 ± 0.983	97.82 ± 2.09
After $(NH_4)_2SO_4$ precipitation	1	2.473 ± 0.301	2.473 ± 0.301	23.28 ± 1.60
Acidic Extract				
Before Extraction	50	0.212 ± 0.016	10.609 ± 0.797	100 ± 0.00
After Extraction	50	0.090 ± 0.013	4.495 ± 0.656	42.82 ± 9.01
After $(NH_4)_2SO_4$ precipitation	1	2.414 ± 0.300	2.414 ± 0.300	22.85 ± 3.52

All experiments were performed in triplicate; All values were in mean ± standard deviation; *Recovery = Total Protein Before Extraction / [Total Protein After Extraction or Total Protein After $(NH_4)_2SO4$ precipitation] x 100 %.

further verifying the presence of antibacterial activity in the mucus extract. The acidic extracts showed significantly greater IZD against *S. braenderup* ATCC BAA 664 than that of their 0.8% acetic acid negative control (p-value = 0.007) which suggests that the fish mucus extracts play a more significant role in antibacterial activity. The MIC tests were conducted in triplicate against *S. braenderup* ATCC BAA 664, yielding a MIC value of 0.302 ± 0.037 mg/ml.

On the other hand, significantly smaller IZD values were observed against *B. cereus* ATCC 33019 (p-value = 0.033) and *S. flexneri* ATCC 12022 (p-value = 0.007) indicating that the negative controls exhibited a stronger antibacterial effect. Although the acidic extracts exhibited a broad spectrum of antibacterial activities against the other 13 tested bacterial strains, their IZD values were insignificant compared to the 0.8 % acetic acid negative control. Therefore, there is insufficient evidence to conclude that the observed activity was demonstrated by acidic mucus extracts or their negative controls.

The protein profiles of the aqueous and acidic extracts exhibited protein bands with similar weights but different intensities. Both mucus extracts displayed several protein bands at 24 kDa, 40 kDa, 50 kDa, 56 kDa and 66 kDa (Fig. 1). However, protein bands in the range of 50 kDa to 70 kDa were more prominent in acidic extracts. The most predominant five bands from SDS-PAGE of the acidic mucus extracts were selected, excised, and subjected to for protein sequencing by LCMS/MS (Proteomic International). The LC-MS/MS analysis data was compared with the UniProt database specific to the fish class Actinopterygii, resulting in the identification of 64 unique proteins out of the initial 155 protein hits and the total sequence coverage for each protein was calculated (Fig. 2; Full description in Supplementary Table 1). Notably, among these proteins, 18 were previously reported in the epidermal mucus of other fish species.

Discussion

Mucus protein concentration and protein recovery

While almost all the protein contents were recovered in the aqueous extracts, only less than half was recovered in the acidic extracts. This result is not surprising as visible pellets (sample loss) were observed during the centrifugation step of the acidic extraction, unlike during the aqueous extraction. The differing results may be due to variations in the mucus composition which react differently to the extraction methods to affect their solubility in different solvents, thereby varying protein concentrations between the aqueous and acidic extracts. A previous study has shown that epidermal mucus production can be influenced by stress factors such as handling, starvation or confinement (Helfman et al. 2009). Further-

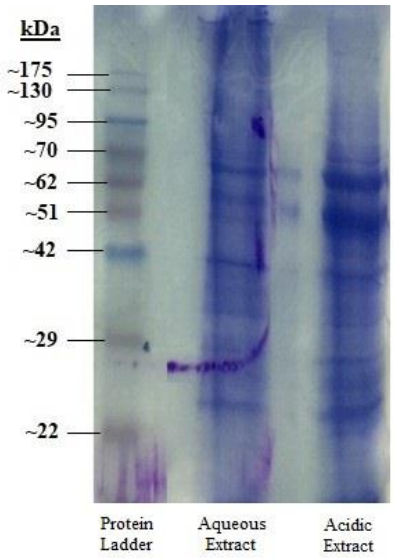

Fig. 1 SDS-PAGE presenting protein profile of the mucus extracts of *Barbodes sealei*. Samples were loaded onto a 4% stacking and 12% resolving acrylamide gel. The staining reagent used was Coomassie Brilliant Blue R-250 (BioRad).

more, the composition of fish mucus can also be influenced by endogenous (sex and developmental stage) and exogenous factors (stress, temperature, pH or infections) (Esteban 2012; Reverter et al. 2018). The present study demonstrated a relatively low protein recovery (< 25 %) for both the aqueous and acidic extracts. To minimise the inevitable losses incurred at every purification step, Doonan and Cutler (2003) recommended reducing the number of purification steps to the barest. Besides varying mucus compositions, which can alter the extraction products, the transfer of samples during preparation may also significantly influence protein loss.

Antibacterial activities of epidermal mucus extracts of *B. sealei*

In this study, aqueous extracts of *B. sealei* were found to be inactive against all the tested bacterial strains. This finding is consistent with a previous extensive review conducted by Lee et al. (2020), which reported the absence of antibacterial activity in aqueous extracts from more than 20 fish species (Hellio et al. 2002; Subramanian et al. 2008b; Subhashini et al. 2013; Katra et al. 2016; Al-Rasheed et al. 2018). In a study by Subramanian et al. (2007), the aqueous skin mucus extracts of seven distinct marine fish species, namely Arctic char, brook trout, koi carp, striped bass, haddock, Atlantic cod, and hagfish, were characterised, confirming the presence of various hydrolytic enzymes such lysozyme and proteases. These enzymes have been reported to exhibit antimicrobial properties in the fish mucus (Aranishi 2000; Smith et al. 2000). Furthermore, studies on organic skin mucus extracts from three freshwater fishes, namely common carp, mrigal and rohu, revealed varying lysozyme and protease activity, with mrigal exhibiting the highest activity and stronger bactericidal effect (Sridhar et al. 2021). The absence of antibacterial activity in the extract may be attributed to unfavourable incubation conditions (temperature or pH) that lead to enzyme inactivation or to insufficient enzyme concentrations that produce negligible antibacterial activity.

During the preliminary screening stage, the acidic extracts exhibited a wide spectrum of antibacterial activities against 14 out of 16 tested bacterial strains. The preparation of the acidic extracts involved the use of acetic acid solvent and short-minute heat treatment, which targeted cationic low molecular weight proteins, resulting in an extract enriched with acid-soluble proteins and peptides (Subramanian et al. 2008b; Manikantan et al. 2016). The use of heat treatment in low concentrations of acetic acid for a brief period can enhance the solubility of cationic proteins and peptides due to their hydrophilic and thermally stable nature (Nigam et al. 2015). It can also selectively inactivate proteolytic enzyme activity that may cause degradation of these cationic peptides (Cole and Ganz 2000). It has been suggested that these acid-soluble proteins play a crucial role in the defensive mechanism, exhibiting broad-spectrum potent antibacterial activities (Hancock and Diamond 2000; Brinchmann 2016). Unlike an aqueous extraction, an acidic extraction pro-

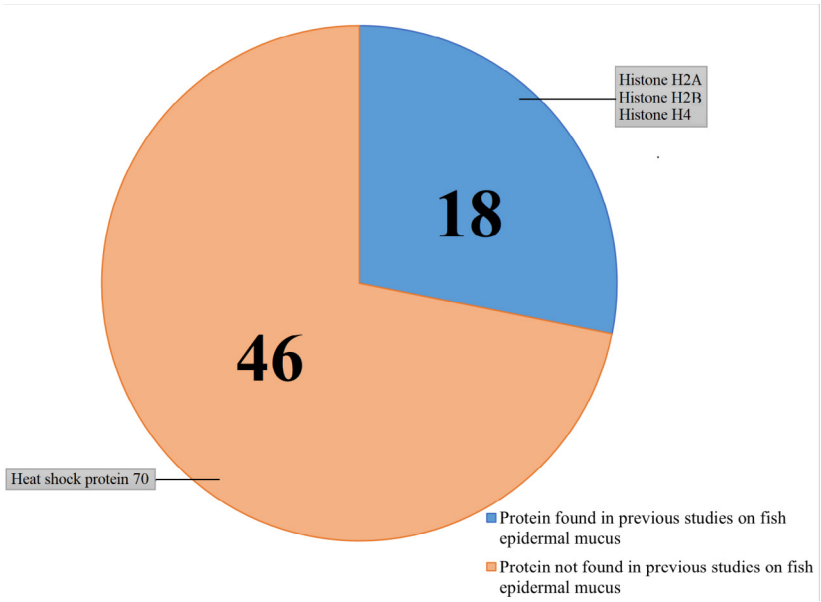

Fig. 2 The number of proteins identified via LC-MS/MS from the acidic mucus extract of *Barbodes sealei*

duces insoluble pellets that are subsequently excluded from the experiment. This is consistent with the Bradford protein essay results, which yielded almost 60 % protein loss from the acidic extraction. Thus, the remaining acid-soluble fraction is believed to contain the cationic peptides, which are purer and free from interference by other proteolytic enzymes, responsible for the antibacterial activity (Li et al. 2007).

This study observed that the acidic negative control, which consisted of 0.8% acetic acid, also exhibited similar activities compared to the acidic mucus extracts. The results were not surprising, as previous studies had shown that acetic acid can exhibit antibacterial activity even at concentrations as low as 0.166 % (Fraise et al. 2013; Wali and Abed 2019). In fact, acetic acid was well established as a disinfectant due to its ability to inhibit a wide range of bacterial pathogens, including those tested in this study, such as *S. aureus*, *E. coli*, and *P. aeruginosa* (Ryssel et al. 2009; Cortesia et al. 2014; Halstead et al. 2015). This study demonstrated that the acidic mucus extracts exhibited a significantly higher IZD against *Salmonella braenderup* compared to its acidic negative control, thereby verifying that the mucus extract, rather than the sole effect of an acidic solvent, was responsible for the observed inhibition zone.

Interestingly, in the presence of mucus extracts, *B. cereus* and *S. flexneri* exhibited better growths, as indicated by lower IZDs, compared to their respective negative controls. Similarly, the mucus extract of Gilthead seabream was reported to have caused the overgrowth of *B. subtilis* (Guardiola et al. 2014). Minniti and co-workers (2019) also found that *Vibrio* sp. and *Pseudoalteromonas* sp. could thrive in the presence of salmon mucus by using the latter as a source of nutrients. Besides, the protein content of fish epidermal mucus may also be a potential nutrient source for enhanced bacterial growth, despite the mucus being widely known for its antimicrobial properties (Smith and Fernandes 2009). It is believed that the defence mechanism of skin mucus in healthy fish against bacterial invasion may differ and needs to be explored.

The positive control (5 µg Ciprofloxacin) used in this study exhibited a broad-spectrum antibacterial activity against all 16 tested bacterial strains. Ciprofloxacin is a commercial antibiotic that contains pure compounds, which are well studied and tested for their antimicrobial activities, unlike the mucus extracts that are still in the screening stage and may require further isolation, purification, and characterisation. Despite having a higher protein concentration of more than 2 mg/ml, the mucus extracts did not exhibit greater antibacterial activities compared to the positive control, just like in a study by Elavarasi et al. (2013) where the protein concentration of walking catfish extract was lower than that of Mozambique tilapia, yet

Table 2. Antibacterial screening of the epidermal mucus acidic extracts* of *Barbodes sealei* against 16 selected bacterial strains

	Inhibition Zone Diameter (IZD) in mm				IZD in mm
Bacterial Strain	Acidic Extract	Negative Control	p-value	Note	Positive Control
Gram-positive					
Bacillus cereus ATCC 33019	12.16 ± 1.10	14.91 ± 0.10	0.033	Control >Extract	24.31 ± 0.79
Listeria monocytogenes ATCC 7644	- ve	-ve	NA	NA	20.55 ± 0.56
Staphylococcus aureus ATCC 25923	8.39 ± 0.42	8.16 ± 0.48	0.565	NS	20.34 ± 0.46
Gram-negative	9.08 ± 0.84	9.83 ± 1.94	0.574	NS	17.37 ± 0.89
Aeromonas hydrophila PRP 012	9.15 ± 1.24	10.13 ± 0.34	0.260	NS	21.32 ± 1.02
Escherichia coli O157:H7	8.95 ± 0.44	8.52 ± 1.37	0.626	NS	14.28 ± 0.60
Klebsiella pneumoniae PRP 010	9.03 ± 1.41	8.19 ± 0.63	0.400	NS	25.24 ± 0.99
Pseudomonas aeruginosa ATCC 27853	10.73 ± 0.16	9.03 ± 0.56	0.007	Extract >Control	29.38 ± 2.14
Salmonella braenderup ATCC BAA 664	9.98 ± 1.24	9.77 ± 1.89	0.878	NS	25.44 ± 0.66
Salmonella enteritidis ATCC 13036	8.92 ± 1.95	8.65 ± 1.25	0.850	NS	22.24 ± 2.11
Salmonella typhi ATCC 14028	10.63 ± 0.75	9.48 ± 0.02	0.057	NS	20.39 ± 0.87
Salmonella typhimurium	8.01 ± 1.04	9.01 ± 0.62	0.226	NS	16.60 ± 0.65
Shigella boydii ATCC 9207	9.35 ± 0.57	11.42 ± 0.42	0.007	Control >Extract	14.07 ± 0.28
Shigella flexneri ATCC 12022	9.77 ± 1.63	10.11 ± 1.01	0.776	NS	26.32 ± 1.04
Shigella sonnei ATCC 25931	7.50 ± 0.61	8.30 ± 0.78	0.234	NS	25.79 ± 1.58
Vibrio cholerae	-ve	-ve	NA	NA	29.09 ± 0.77

All experiments are done in triplicates; All values were in mean ± standard deviation; IZD (Inhibition Zone Diameter) includes 6 mm disc diameter; -ve indicates no clear zone of inhibition observed (Absence of antibacterial activity); Negative Control = ~0.8 % acetic acid; Positive Control = Ciprofloxacin disc (5 µg); NA indicates that t-test is not performed; NS indicates no significant difference between mucus extract and its negative control (Absence of antibacterial activity by mucus extract) while data in Bold indicates significant difference between mucus extract and negative control (Presence of antibacterial activity by mucus extract). * Only acid extract data is shown here. Aqueous extracts were inactive against all bacterial strains tested (Data not shown)

the former exhibited better bactericidal activities. This suggests that protein concentration is not a simple measure of antibacterial activity in fish skin mucus extracts. A high protein contents not only increases the chance of having more antibacterial proteins in the extracts, but may also indicate the presence of inert contaminants that do not contribute to any activity (Al-Rasheed et al. 2018) and may potentially dilute the effect of any active compound.

To further characterise the antibacterial activity of the active mucus extracts, MIC tests were conducted. While there have been reports on the antibacterial activity of fish epidermal mucus against *Salmonella* sp., this – as far as we know - is the first report of minimal inhibitory activities against *S. braenderup*. However, the MIC values obtained contradict the findings of Vennila et al. (2011) where the acidic mucus extract of marine stingray inhibited the growth of another *Salmonella* sp with much lower MIC values (16-32 µg/ml). Elsewhere, Rao et al. (2015) reported that acidic mucus extract from bagrid catfish inhibited bacterial growth at a concentration as low as 23.91 µg/ml, while Subramanian et al. (2008b) extracted the skin mucus from various species, such as brook trout, haddock and hagfish, using an acidic solvent and achieved MIC values ranging from 21 to 273 µg/ml against *P. aeruginosa*. This confirmed that the protein contents of fish skin mucus were not positively correlated with the antibacterial activity exhibited and might vary among different fish species and extraction methods. Schuurmans et al. (2009) stated that the standardised protocol followed by different laboratories might have variations in the duration of measurement, culture density, and the parameters used to determine growth, leading to up to 8-fold differences in MIC values. While this does not undermine the reliability of reports adhering to the same protocol, it can make it challenging to compare MIC values across studies and may misrepresent the true MIC value of a given set of microorganisms and fish mucus extract.

The preliminary antibacterial screening conducted in this study indicates that the acidic extract of *B. sealei* exhibited antimicrobial activity, suggesting that it has the potential to be a valuable source of antimicrobial compounds. However, further studies will be required to purify and characterise the antibacterial components in fish skin mucus.

Antimicrobial proteins (AMP) in acidic extract

In comparison to relevant existing literature, our study reported 18 out of 64 identified proteins in the epidermal mucus of other fish species and four antibacterial proteins, namely Histone H2A, Histone H2B, Histone H4, and Heat shock protein 70. (See Fig. 2 and Table 3).

The study of antimicrobial peptides or proteins (AMPs) gained momentum in the 1980s with the discovery of insect cecropins (Steiner et al. 1981), human α-defensins (Selsted et al. 1985) and amphibians magainins (Zasloff 1987). Since then, the database of identified AMPs has been steadily expanding, with over 3000 antimicrobial peptides have been isolated and described across various living species. While the majority of AMPs are found in animals (Wang et al. 2016), fish peptides represent only about 5 % of the total (Masso-Silva and Diamond 2014).

Fish, inhabiting diverse aquatic environments encompassing both freshwater and marine habitats, are constantly exposed to fluctuations in salinity, temperature, pH, and a wide range of microbial pathogens. As a result, fish have evolved an impressive repertoire of AMPs, including cathelicidins, defensins, hepcidins, histone-derived peptides, and piscidins, which exhibit remarkable diversity in their sequences, structures, and functions (Masso-Silva and Diamond 2014). These fish AMPs have demonstrated their efficacy in combating a broad spectrum of pathogens, even in challenging conditions. Importantly, they exhibit high selectivity and potency against pathogens, while showing minimal toxicity towards host tissues and mammalian cells (Kim et al. 2010). This unique combination of diversity, selectivity, and safety makes fish AMPs highly promising for therapeutic applications in various environments, including aquaculture and human healthcare settings.

Recent advancements in genomic and proteomic techniques have facilitated the identification and characterisation of a growing number of fish AMPs. Through the use of proteomic technology such as LC-MS/MS, the major proteins present in the active epidermal mucus extracts in the study were successfully identified. The observed activity of the acidic mucus extract can be attributed to these fish AMPs.

Histones are highly conserved and ubiquitous proteins found in the nuclei of all eukaryotes. This family of proteins comprises linker histones (H1 and H5) and core histones (H2A, H2B, H3 and H4) which are re-

Table 3 Proteins identified via LC-MS/MS and total sequence coverage from the acidic mucus extract of *Barbodes seale*

Protein sequence coverage (%)	Protein jame	Reported in fish epidermal mucus
18.99	78 kDa glucose-regulated protein	*Sparus aurata* (Sanahuja and Ibarz 2015; Pérez-Sánchez et al. 2017) *Salmo salar* (Jensen et al. 2014)
1.75	Abelson helper integration site 1	NA
15.47	Actin, cytoplasmic 1	*Gadus morhua* (Rajan et al. 2011) *Cyclopterus lumpus* (Patel and Brinchmann 2017; Patel et al. 2019) *Dicentrarchus labrax* (Cordero et al. 2015) *Salmo salar* (Fæste et al. 2020) *Sparus aurata* (Cordero et al. 2017; Pérez-Sánchez et al. 2017)
10.97	Actin, cytoplasmic 2-like	NA
2.48	Alanine--tRNA ligase	
2.69	Alpha-1-antitrypsin	*Acipenser oxyrhynchus oxyrhynchus* (Murphy et al. 2020)
0.76	Alpha-2-macroglobulin isoform X1	
1.18	Anaphase-promoting complex subunit 5	
0.62	ATPase family AAA domain-containing protein 5-like	NA
0.67	Centrosomal protein 350	
5.82	Coiled-coil domain-containing 18-like isoform X1	
1.52	Complement C3-like protein	*Pelteobagrus fulvidraco* (Xiong et al. 2020)
2.26	Echinoderm microtubule-associated-like 2 isoform X1	
1.49	EMAP like 2	
10.97	Gelsolin	
1.76	Gelsolin Actin-depolymerizing factor	
7.50	Gelsolin-like	
4.17	Gelsolin-like domain-containing protein	NA
7.64	gelsolin-like isoform X1	
8.33	Glutathione S-transferase omega	
2.93	Guanine nucleotide binding protein (G protein), alpha 15 (Gq class), tandem duplicate 4	
12.58	Heat shock 70 kDa protein-like	
21.57	Heat shock cognate 70	
20.80	Heat shock cognate 70 kDa protein	*Cathorops spixii* (Ramos et al. 2012) *Gadus morhua* (Magnadóttir et al. 2018) *Larimichthys crocea* (Ao et al. 2015) *Sparus aurata* (Jurado et al. 2015) *Salmo salar* (Provan et al. 2013; Jensen et al. 2014)
21.08	Heat shock cognate 70 kDa protein-like	NA
26.19	Heat shock cognate 71 kDa protein	*Cathorops spixii* (Ramos et al. 2012) *Larimichthys crocea* (Ao et al. 2015) *Sparus aurata* (Cordero et al. 2017; Pérez-Sánchez et al. 2017)
24.20	Heat shock cognate 71 kDa protein-like	*Boleophthalmus pectinirostris* (Liu et al. 2019)
7.23	Heat shock cognate protein 70	
16.51	Heat shock protein 70 (Fragment)	
7.13	Heat shock protein family A (Hsp70) member 2	
18.31	Heat shock protein family A (Hsp70) member 8	NA
16.49	Heat shock protein Hsc70	
24.77	Heat-Shock Cognate 70kd Protein (Fragment)	
15.38	Hemoglobin subunit alpha	
8.54	Hemopexin	*Sparus aurata* (Pérez-Sánchez et al. 2017)
8.66	Histone H2A	*Channa striata* (Kwan and Ismail 2018) *Cyclopterus lumpus* (Patel et al. 2019) *Salmo salar* (Fæste et al. 2020) *Sparus aurata* (Cordero et al. 2017) *Oncorhynchus mykiss* (Fernandes et al. 2002)
7.86	Histone H2A type 2-A (Fragment)	NA
18.33	Histone H2B	Cyclopterus lumpus (Patel et al. 2019) Gadus morhua (Bergsson et al. 2005) Salmo salar (Fæste et al. 2020) Sparus aurata (Cordero et al. 2017)
14.81	Histone H3	*Cirrhinus mrigala* (Nigam et al. 2015) *Gadus morhua* (Magnadóttir et al. 2018) *Myxine glutinosa* (Subramanian et al. 2008a)
9.20	Histone H3-like	*Pelteobagrus fulvidraco* (Xiong et al. 2020)
17.86	Histone H4	*Channa striata* (Kwan and Ismail 2018) *Cyclopterus lumpus* (Patel et al. 2019) *Dicentrarchus labrax* (Cordero et al. 2015) *Sparus aurata* (Cordero et al. 2017)
2.75	IF rod domain-containing protein	*Sparus aurata* (Pérez-Sánchez et al. 2017)
6.08	Ig-like domain-containing protein	NA
6.90	Inducible heat shock protein 70	
5.74	Intermediate filament protein ON3	
2.86	Intermediate filament protein ON3-like	NA
5.45	Keratin 4	
5.49	Keratin, type II cytoskeletal 8-like	*Boleophthalmus pectinirostris* (Liu et al. 2019) *Dicentrarchus labrax* (Cordero et al. 2015) *Pelteobagrus fulvidraco* (Xiong et al. 2020) *Sparus aurata* (Sanahuja et al. 2019)

Table 3 Continued

16.47	L-lactate dehydrogenase	*Carassius auratus gibelio* (Jiang et al. 2019) *Channa striata* (Kwan and Ismail 2018)
3.63	Major vault protein	*Salmo salar* (Valdenegro-Vega et al. 2014)
1.48	Pol-like protein	
7.24	Putative histone H2B type 2-E-like	
4.08	Putative threonine-rich GPI-anchored glyco isoform X2	
3.77	S-adenosyl-L-homocysteine hydrolase NAD binding domain-containing protein	NA
2.35	Sarcosine dehydrogenase	
10.26	Scinderin like b	
7.36	Scinderin-like a	
6.66	Serotransferrin	*Channa striata* (Kwan and Ismail 2018) *Gadus morhua* (Magnadóttir et al. 2018)
1.69	Si:dkey-65b12.6 (Fragment)	
3.31	Threonyl-tRNA synthetase	
0.96	Transmembrane protein 132D	NA
1.80	UmuC domain-containing protein	
3.50	Warm-temperature-acclimation-associated 65-kDa protein	
3.64	WD repeat domain 1	

NA indicates that the protein is not reported in fish epidermal mucus elsewhere.

sponsible for the formation of nucleosomes. Traditionally, they were believed to provide structural support for DNA and regulate gene transcription (Parseghian and Luhrs 2006). However, histones have emerged as a promising source of AMPs through numerous studies over the years.

Core histone H2A has been found to possess potent antibacterial properties, both as a full-length protein or derived peptide fragments (Doolin et al. 2020). Full-length H2A purified from skin exudates of rainbow trout exhibited activity against several Gram-positive bacteria at a maximum concentration of 16 μg / ml (Fernandes et al. 2002). Similarly, several truncated N-terminal fragments of H2A from various aquatic organisms have also exhibited broad-spectrum antibacterial activity. Such peptide fragments include abhisin from disk abalone (De Zoysa et al. 2009), buforins from various amphibians and clam species (Li et al. 2007; Cho et al. 2009; Muñoz-Camargo et al. 2018), hipposin from Atlantic halibut (Birkemo et al. 2003), parasin I from Japanese common catfish (Park et al. 1998), and several unidentified fragments from shrimps, crabs and fishes (Patat et al. 2004; Chen et al. 2015; Ma et al. 2017; Sruthy et al. 2019). In most cases, these fragments were generated through proteolytic cleavage.

The antimicrobial properties of H2B were initially reported in murine macrophages by Hiemstra et al. (1993). In the following decade, more researchers isolated H2B from gills, skin, and surface mucus of various fish species (Robinette et al. 1998; Noga et al. 2001; Bergsson et al. 2005), as well as from the skin of Schlegel's green tree frog (Kawasaki et al. 2003) and haemocytes of Pacific white shrimp (Patat et al. 2004). These studies demonstrated the inhibitory effects of H2B against many pathogenic bacterial strains. Notably, the potent activity of H2B against the fish pathogen *Aeromonas hydrophilia* suggests its crucial role in fish immunity (Robinette et al. 1998). While research on core histone H4 has been relatively limited, this histone has been purified from shrimp haemocytes (Patat et al. 2004) and secretions of human sebocytes (Lee et al. 2009), both of which have been reported to exhibit potent antimicrobial activity against Gram-positive and Gram-negative bacteria (Knappe et al. 2009). Lee et al. (2009) also reported the enhancer role of histone H4 in increasing the antimicrobial effect of sebum-free fatty acids. This finding suggests that histones may have alternative roles in combating bacterial infections beyond their specialized AMP function.

Heat shock proteins (HSPs) are highly conserved stress-response proteins that are found in various organisms, including fish (Morimoto and Santoro 1998; Demeke and Tassew 2016). Apart from heat stress, they can be upregulated in response to different stress stimuli, such as acidosis, hypoxia, ischaemia, microbial damage, or protein degradation (Roberts et al. 2010). In general, HSPs are grouped based on their molecular masses, including low molecular weight heat shock proteins (>47 kDa), Hsp70 (68–73 kDa) and Hsp90 (85–90 kDa). Among these, Hsp70 plays significant roles in fish health, particularly in the development of specific or non-specific immune responses to bacterial and viral infections. The antibacterial significance of Hsp70 was first revealed by Forsyth et al. (1997) who observed increased Hsp70 levels over a 63-day period in coho salmons infected with *Renibacterium salmoninarum*. Roberts et al. (2010) reported that elevated Hsp70 synthesis in salmon and gilthead seabream enhanced by a chemical inducer

called TEX-OE®, substantially increased their survivability when challenge with *Vibrio*. Moreover, platy fish treated with intra-coelomal injection of two bacterial HSPs, DnaK and GroEL (equivalent to Hsp70 and Hsp60), along with a non-lethal heat shock, survived *Yersinia ruckeri* infections (Ryckaert et al. 2010). Although the exact mode of action of Hsp70 in bacterial inhibition is not fully understood, these findings confirmed the importance of its bactericidal role in fish. In 2013, Taniguchi et al. demonstrated the antibacterial properties of Hsp70-18, a potent octadecapeptide derived from rice Hsp70 (*Oryza sativa* L. japonica). They elucidated the mechanism of action, showing that Hsp70-18 inhibits the growth of the Gram-negative *Porphyromonas gingivalis* ATCC 33277. The strength of the antibacterial activity was observed to be closely correlated with the degree of cell membrane disruption.

In the present study, it is postulated that the antibacterial proteins identified from the acidic extract of *B. sealei,* namely Histone H2A, Histone H2B, Histone H4 and Heat shock protein 70, play a major role in the exhibited *in vitro* antibacterial activity. However, the exact roles of these antibacterial proteins are still unknown, and it is unclear whether their activity is influenced by interactions with other proteins or if each protein can independently act as an antimicrobial agent. To further elucidate their biological and biochemical roles, as well as the mechanisms of their antimicrobial activity, future experiments should focus on isolating and purifying the proteins of interest. By precisely characterising these proteins, their function can be better understood, allowing for more accurate assessments of fish health and disease monitoring.

Conclusion

The native freshwater fish species of Borneo, including *Barbodes sealei*, have been relatively understudied. However, this Bornean endemic species hold great potential as a source of biologically active compounds. In the present study, two types of mucus protein extracts were successfully purified and concentrated from *B. sealei*. The difference in protein contents between the aqueous and the acidic mucus extracts could be attributed to their varying solubility in different solvents. The acidic mucus extract from *B. sealei* exhibited inhibitory effects against the human pathogen *S. braenderup* ATCC BAA 664. Studies suggested that acidic extraction could enhance the solubility of cationic antimicrobial peptides to produce purer compounds free from proteolytic enzymes that might compromise their antibacterial effect.

The present results indicated that higher protein contents did not necessarily translate to greater antibacterial activities. The relevant existing literature was reviewed in the light of the proteins predominantly identified in the active epidermal mucus extracts used in the study. Among all the proteins identified, Histone H2A, H2B, H4, and Heat shock protein 70 were reported to possess antibacterial properties. However, further fractionation, purification, and characterisation of these proteins are needed for a deeper understanding of their mechanisms of action within fish epidermal mucus. This current study has reinforced the significance of fish epidermal mucus as an antimicrobial agent and has opened up new avenues for exploring the antimicrobial potential of freshwater fish epidermal mucus. It represents a low-cost and sustainable source that holds promise for the isolation and discovery of novel biologically active compounds.

Competing interests The authors declare that they have no competing interests.

Authors' contributions YL have made a substantial contribution to the concept and design of all the experiments as well as acquisition, analysis and interpretation of data for the article. JZL have contributed partly for the experiment. LMB, BS, and NSN have made a great contribution in experimental designs and helped to revise the article critically for important intellectual content. YLC have contributed substantially in the concept, design, and the direction of the research, analysis and interpretation of data, refined the writing style and language as well as helped to finalise the manuscript. All authors read and approved the final manuscript.

Acknowledgements This project was fully funded by Tun Ahmad Zaidi Chair Grant (F07/TZC/1592/2017) awarded to YLC. Collection of fish samples were made under permit granted by Sarawak Forestry Corporation (Permit No. NPW.907.4.4(JLD.14)-287 and Park Permit No. WL111/2017). The authors would like to thank Faculty of Resource Science and Technology, UNIMAS for the administrative supports. The authors thank anonymous reviewers who have been making great contribution in the reviewing of this manuscript.

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Supplementary Table 1 Protein identified via LC-MS/MS and total sequence coverage from the acidic mucus extract of *Barbodes sealei.*

db|UniqueIdentifier|EntryName	Protein sequence Coverage (%)	Protein name	Organism name	Gene name
BAND 1				
tr|B5X872|B5X872_SALSA	15.47	Actin, cytoplasmic 1	*Salmo salar*	ACTB
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	7.24	Putative histone H2B type 2-E-like	*Scophthalmus maximus*	SMAX5B_012257
tr|A0A0P7UMM5|A0A0P7UMM5_SCLFO	10.97	Actin, cytoplasmic 2-like	*Scleropages formosus*	Z043_118570
tr|A0A3N0Y8D6|A0A3N0Y8D6_ANAGA	15.00	Histone H4	*Anabarilius grahami*	DPX16_9602
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	14.81	Histone H3	*Cyprinodon variegatus*	NA
tr|A0A2I4CE20|A0A2I4CE20_9TELE	9.20	Histone H3-like	*Austrofundulus limnaeus*	LOC106527799
tr|A0A3B1J9L7|A0A3B1J9L7_ASTMX	15.38	Hemoglobin subunit alpha	*Astyanax mexicanus*	NA
tr|A0A3B4VLV2|A0A3B4VLV2_SERDU	5.49	Keratin, type II cytoskeletal 8-like	*Seriola dumerili*	NA
tr|M3ZFR9|M3ZFR9_XIPMA	2.86	Intermediate filament protein ON3-like	*Xiphophorus maculatus*	NA
tr|A0A3P8QTL6|A0A3P8QTL6_ASTCA	2.35	Sarcosine dehydrogenase	*Astatotilapia calliptera*	NA
tr|A0A3P9J2C2|A0A3P9J2C2_ORYLA	5.74	Intermediate filament protein ON3	*Oryzias latipes*	NA
tr|A0A4U5UXM5|A0A4U5UXM5_COLLU	0.85	Gelsolin Actin-depolymerizing factor	*Collichthys lucidus*	D9C73_013700
tr|A6QL59|A6QL59_DANRE	8.66	Histone H2A	*Danio rerio*	hist1h2a6
tr|A0A498NJJ0|A0A498NJJ0_LABRO	4.08	Putative threonine-rich GPI-anchored glyco isoform X2	*Labeo rohita*	ROHU_004713
tr|A0A2R8Q0V6|A0A2R8Q0V6_DANRE	5.45	Keratin 4	*Danio rerio*	krt4
tr|A0A146ZCQ4|A0A146ZCQ4_FUNHE	18.33	Histone H2B	*Fundulus heteroclitus*	NA
tr|A0A3G9CN67|A0A3G9CN67_CYPCA	8.33	Glutathione S-transferase omega	*Cyprinus carpio*	NA
tr|A0A146UBU9|A0A146UBU9_FUNHE	15.88	Histone H2B (Fragment)	*Fundulus heteroclitus*	NA
tr|A0A3Q3VLK8|A0A3Q3VLK8_MOLML	0.96	Transmembrane protein 132D	*Mola mola*	TMEM132D
tr|F1QJS8|F1QJS8_DANRE	1.69	Si:dkey-65b12.6 (Fragment)	*Danio rerio*	si:dkey-65b12.6
tr|A0A060VZ29|A0A060VZ29_ONCMY	2.75	IF rod domain-containing protein	*Oncorhynchus mykiss*	GSONMT00081034001
tr|H3DDD8|H3DDD8_TETNG	2.93	Guanine nucleotide binding protein (G protein), alpha 15 (Gq class), tandem duplicate 4	*Tetraodon nigroviridis*	NA
tr|Q76IL7|Q76IL7_DANRE	1.48	Pol-like protein	*Danio rerio*	ORF2
tr|A0A484DCQ2|A0A484DCQ2_PERFV	1.80	UmuC domain-containing protein	*Perca flavescens*	EPR50_G00053750
tr|A0A3B3HRV0|A0A3B3HRV0_ORYLA	6.08	Ig-like domain-containing protein	*Oryzias latipes*	NA

Supplementary Table 1 Continued

tr|A0A3Q2GKU3|A0A3Q2GKU3_CYPVA BAND 2	1.18	Anaphase-promoting complex subunit 5	*Cyprinodon variegatus*	ACTB
tr|A0A3N0XEC2|A0A3N0XEC2_ANAGA	10.97	Gelsolin		DPX16_20242
tr|A0A498M1X6|A0A498M1X6_LABRO	5.82	Coiled-coil domain-containing 18-like isoform X1	*Anabarilius grahami*	ROHU_028433
tr|A0A3Q3IH74|A0A3Q3IH74_MONAL	7.08	Gelsolin-like	*Labeo rohita*	NA
tr|A0A3Q1IYL6|A0A3Q1IYL6_ANATE	7.04	Scinderin like b	*Monopterus albus*	NA
tr|A0A0R4IQ11|A0A0R4IQ11_DANRE	7.36	Scinderin-like a	*Anabas testudineus*	scinla
tr|A0A444U3J4|A0A444U3J4_ACIRT	4.25	Gelsolin	*Danio rerio*	EOD39_8512
tr|A0A1S3PAL0|A0A1S3PAL0_SALSA	5.97	Gelsolin-like	*Acipenser ruthenus*	LOC106584171
tr|G3PSP8|G3PSP8_GASAC	10.26	Scinderin like b	*Salmo salar*	NA
tr|A0A2I4CLF4|A0A2I4CLF4_9TELE	7.64	Gelsolin-like isoform X1	*Gasterosteus aculeatus*	LOC106529836
tr|W5UTS7|W5UTS7_ICTPU	8.19	Gelsolin	*Austrofundulus limnaeus*	GSN
tr|A0A3Q0SV12|A0A3Q0SV12_AMPCI	5.28	Scinderin like b	*Ictalurus punctatus*	NA
tr|A0A3B4DAN9|A0A3B4DAN9_PYGNA	5.83	Gelsolin-like	*Amphilophus citrinellus*	NA
tr|A0A3Q2V687|A0A3Q2V687_HAPBU	7.50	Gelsolin-like	*Pygocentrus nattereri*	NA
tr|B5X872|B5X872_SALSA	15.47	Actin, cytoplasmic 1	*Haplochromis burtoni*	ACTB
tr|A0A3P8XCQ1|A0A3P8XCQ1_ESOLU	5.58	Gelsolin	*Salmo salar*	NA
tr|A0A3B3DPM0|A0A3B3DPM0_ORYME	5.42	Gelsolin-like	*Esox lucius*	NA
tr|A0A3B3RPQ6|A0A3B3RPQ6_9TELE	4.03	Gelsolin-like	*Oryzias melastigma*	NA
tr|A0A1S3QZ97|A0A1S3QZ97_SALSA	6.39	Gelsolin-like	*Paramormyrops kingsleyae*	LOC106598932
tr|A0A4U5UXM5|A0A4U5UXM5_COLLU	1.76	Gelsolin Actin-depolymerizing factor	*Salmo salar*	D9C73_013700
tr|A0A3B4BU39|A0A3B4BU39_PYGNA	5.98	Gelsolin-like	*Collichthys lucidus*	NA
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	4.82	Putative histone H2B type 2-E-like	*Pygocentrus nattereri*	SMAX5B_012257
tr|A0A3B1J9L7|A0A3B1J9L7_ASTMX	15.38	Hemoglobin subunit alpha	*Scophthalmus maximus*	
tr|A0A0P7UMM5|A0A0P7UMM5_SCLFO	10.97	Actin, cytoplasmic 2-like	*Astyanax mexicanus*	Z043_118570
tr|A0A3B3THM5|A0A3B3THM5_9TELE	17.86	Histone H4	*Scleropages formosus*	NA
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	14.81	Histone H3	*Poecilia latipinna*	NA
tr|A0A2I4CE20|A0A2I4CE20_9TELE	9.20	Histone H3-like	*Cyprinodon variegatus*	LOC106527799
tr|H2L816|H2L816_ORYLA	3.31	Threonine--tRNA ligase	*Austrofundulus limnaeus*	LOC101171337
tr|A0A060Y244|A0A060Y244_ONCMY	6.00	Hemopexin	*Oryzias latipes*	GSONMT00038203001
tr|A6QL59|A6QL59_DANRE	8.66	Histone H2A	*Oncorhynchus mykiss*	hist1h2a6
tr|A0A2D0SST5|A0A2D0SST5_ICTPU	0.76	Alpha-2-macroglobulin isoform X1	*Danio rerio*	LOC108277478
tr|A0A146QB50|A0A146QB50_FUNHE	7.86	Histone H2A type 2-A (Fragment)	*Ictalurus punctatus*	NA
tr|A0A437C175|A0A437C175_ORYJA BAND 3	2.18	Uncharacterized protein	*Fundulus heteroclitus* *Oryzias javanicus*	OJAV_G00232420
tr|A0A3N0Z785|A0A3N0Z785_ANAGA	24.13	Heat shock cognate 71 kDa protein		DPX16_10733
tr|A0A1U9X9S4|A0A1U9X9S4_CHACN	26.19	Heat shock cognate 71 kDa protein		NA
tr|W5KA74|W5KA74_ASTMX	21.08	Heat shock cognate 70 kDa protein-like	*Anabarilius grahami*	NA
tr|A0A1I9LXI2|A0A1I9LXI2_ANGMA	21.57	Heat shock cognate 70	*Chanos chanos*	hsc70
tr|A0A3Q3AR85|A0A3Q3AR85_KRYMA	24.20	Heat shock cognate 71 kDa protein-like	*Astyanax mexicanus*	NA
tr|A0A3P8WYY7|A0A3P8WYY7_CYNSE	20.80	Heat shock cognate 70 kDa protein	*Anguilla marmorata*	NA
tr|A0A146NKP1|A0A146NKP1_FUNHE	22.34	Heat shock cognate 71 kDa protein	*Kryptolebias marmoratus*	NA
tr|Q6QIS4|Q6QIS4_PIMPR	19.54	Heat shock cognate 70 kDa protein	*Cynoglossus semilaevis*	HSP70
tr|A0A2U9B4I2|A0A2U9B4I2_SCOMX	19.35	Heat shock cognate 71 kDa protein	*Fundulus heteroclitus*	SMAX5B_004559
tr|A0A3B4CQA3|A0A3B4CQA3_PYGNA	18.31	Heat shock protein family A (Hsp70) member 8	*Pimephales promelas*	NA
tr|A0A3P9AI26|A0A3P9AI26_ESOLU	17.11	Heat shock cognate 70 kDa protein	*Scophthalmus maximus*	NA
tr|A0A3B5AG78|A0A3B5AG78_9TELE	20.74	Heat shock cognate 71 kDa protein-like	*Pygocentrus nattereri*	NA
tr|A0A3Q3MLV6|A0A3Q3MLV6_9TELE	16.87	Heat shock cognate 71 kDa protein-like	*Esox lucius*	NA
tr|A0A3B4UD02|A0A3B4UD02_SERDU	18.13	Heat shock cognate 71 kDa protein-like	*Stegastes partitus*	NA
tr|V9PTF2|V9PTF2_SCHPR	16.49	Heat shock protein Hsc70	*Mastacembelus armatus*	Hsc70
tr|Q6PGX4|Q6PGX4_DANRE	19.01	Heat shock cognate 70	*Seriola dumerili*	hsc70
tr|A0A2P1K697|A0A2P1K697_MYLPI	18.99	78 kDa glucose-regulated protein	*Schizothorax prenanti*	NA
tr|A0A0P7U8Q6|A0A0P7U8Q6_SCLFO	24.77	Heat-Shock Cognate 70kd Protein (Fragment)	*Danio rerio*	Z043_117667
tr|A0A3Q3B3S9|A0A3Q3B3S9_KRYMA	16.36	Heat shock cognate 71 kDa protein	*Mylopharyngodon piceus*	NA
tr|A0A1S3MI49|A0A1S3MI49_SALSA	12.58	Heat shock 70 kDa protein-like	*Scleropages formosus*	LOC106572869
tr|A0A3N0Z6I9|A0A3N0Z6I9_ANAGA	16.85	78 kDa glucose-regulated protein	*Kryptolebias marmoratus*	DPX16_9564
tr|A0A3B3SXK8|A0A3B3SXK8_9TELE	14.80	Heat shock cognate 71 kDa protein-like	*Salmo salar*	NA
tr|A0A3Q1HXW4|A0A3Q1HXW4_ANATE	14.33	Heat shock cognate 70	*Anabarilius grahami*	NA
tr|A0A3B3ZFX4|A0A3B3ZFX4_9GOBI	15.41	Heat shock cognate 70	*Paramormyrops kingsleyae*	NA
tr|A0A3P9MHS2|A0A3P9MHS2_ORYLA	13.46	Heat shock cognate 70	*Anabas testudineus*	NA
tr|A0A3B5KIU0|A0A3B5KIU0_TAKRU	14.59	Heat shock cognate 71 kDa protein-like	*Periophthalmus magnuspinnatus*	LOC101075813
tr|A0A172LPZ7|A0A172LPZ7_TACFU	11.83	78 kDa glucose-regulated protein (Fragment)	*Oryzias latipes*	NA
tr|A8CEI1|A8CEI1_POERE	11.23	78 kDa glucose-regulated protein	*Takifugu rubripes*	NA
tr|A0A2U9C1L7|A0A2U9C1L7_SCOMX	6.90	Inducible heat shock protein 70	*Tachysurus fulvidraco*	SMAX5B_014757
tr|A0A3B3CHS0|A0A3B3CHS0_ORYME	11.82	Heat shock cognate 71 kDa protein-like	*Poecilia reticulata*	NA
tr|A0A315W4Q1|A0A315W4Q1_GAMAF	3.77	S-adenosyl-L-homocysteine hydrolase NAD binding domain-containing protein	*Scophthalmus maximus*	CCH79_00010700
tr|B5X872|B5X872_SALSA	15.47	Actin, cytoplasmic 1	*Oryzias melastigma*	ACTB
tr|Q8JHD1|Q8JHD1_CARAU	6.66	Serotransferrin	*Gambusia affinis*	TF
tr|A0A3Q1EUS7|A0A3Q1EUS7_9TELE	7.13	Heat shock protein family A (Hsp70) member 2	*Salmo salar*	HSPA2
tr|A0A444U3J4|A0A444U3J4_ACIRT	3.23	Gelsolin	*Carassius auratus*	EOD39_8512
tr|A0A0P7UMM5|A0A0P7UMM5_SCLFO	10.97	Actin, cytoplasmic 2-like	*Acanthochromis polyacanthus*	Z043_118570
tr|A0A3N0XEC2|A0A3N0XEC2_ANAGA	5.14	Gelsolin	*Acipenser ruthenus*	DPX16_20242
tr|A0A3B1J9L7|A0A3B1J9L7_ASTMX	15.38	Hemoglobin subunit alpha	*Scleropages formosus*	NA
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	14.81	Histone H3	*Anabarilius grahami*	NA
tr|A0A3N0XQ52|A0A3N0XQ52_ANAGA	6.94	Gelsolin	*Astyanax mexicanus*	DPX16_19757
tr|A0A2I4CE20|A0A2I4CE20_9TELE	9.20	Histone H3-like	*Cyprinodon variegatus*	LOC106527799

Supplementary Table 1 Continued

tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	4.82	Putative histone H2B type 2-E-like	*Anabarilius grahami*	SMAX5B_012257
tr|A0A3B3THM5|A0A3B3THM5_9TELE	17.86	Histone H4	*Austrofundulus limnaeus*	NA
tr|A0A498LU05|A0A498LU05_LABRO	1.52	Complement C3-like protein	*Scophthalmus maximus*	ROHU_010504
tr|I3J0M2|I3J0M2_ORENI	0.67	Centrosomal protein 350	*Poecilia latipinna*	NA
tr|A0A3B4DAN9|A0A3B4DAN9_PYGNA	4.17	Gelsolin-like	*Labeo rohita*	NA
tr|Q52RN6|Q52RN6_RACCA	16.51	Heat shock protein 70 (Fragment)	*Oreochromis niloticus*	NA
tr|A0A3Q2XJ85|A0A3Q2XJ85_HIPCM	0.62	ATPase_AAA_core domain-containing protein	*Pygocentrus nattereri*	NA
tr|Q8UVE7|Q8UVE7_CYPCA	2.84	Serotransferrin	*Rachycentron canadum*	NA
tr|A0A3B4VEP6|A0A3B4VEP6_SERDU	2.48	Alanine--tRNA ligase	*Hippocampus comes*	AARS
tr|A0A3B4BU39|A0A3B4BU39_PYGNA	4.17	Gelsolin-like	*Cyprinus carpio*	NA
tr|W5NC62|W5NC62_LEPOC	1.49	EMAP like 2	*Seriola dumerili*	NA
tr|A0A498MTM3|A0A498MTM3_LABRO	1.15	IF rod domain-containing protein	*Pygocentrus nattereri*	ROHU_021778
tr|A6QL59|A6QL59_DANRE	8.66	Histone H2A	*Lepisosteus oculatus*	hist1h2a6
tr|A0A4U5UXM5|A0A4U5UXM5_COLLU	0.85	Gelsolin Actin-depolymerizing factor	*Labeo rohita*	D9C73_013700
tr|A0A060XJW5|A0A060XJW5_ONCMY	4.17	Gelsolin-like domain-containing protein	*Danio rerio*	GSONMT00034728001
tr|A0A3B3HRV0|A0A3B3HRV0_ORYLA	6.08	Ig-like domain-containing protein	*Collichthys lucidus*	NA
tr|A0A3Q2GKU3|A0A3Q2GKU3_CYPVA	1.18	Anaphase-promoting complex subunit 5	*Oncorhynchus mykiss*	NA
tr|Q5SEP6|Q5SEP6_GRASX	7.27	Histone H3 (Fragment)	*Oryzias latipes*	NA
tr|A0A498LHW8|A0A498LHW8_LABRO	2.26	Echinoderm microtubule-associated-like 2 isoform X1	*Cyprinodon variegatus*	ROHU_011891
BAND 4			*Grammistes sexlineatus*	
tr|A0A096VJY6|A0A096VJY6_EPICO	7.23	Heat shock cognate protein 70	*Labeo rohita*	hsc70
tr|A0A498LX76|A0A498LX76_LABRO	3.63	Major vault protein		ROHU_029253
tr|A0A0A1HAN6|A0A0A1HAN6_9TELE	8.54	Hemopexin		Wap65-1
tr|B5X872|B5X872_SALSA	12.27	Actin, cytoplasmic 1	*Epinephelus coioides*	ACTB
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	14.81	Histone H3	*Labeo rohita*	NA
tr|A0A2I4CE20|A0A2I4CE20_9TELE	9.20	Histone H3-like	*Carassius carassius*	LOC106527799
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	4.82	Putative histone H2B type 2-E-like	*Salmo salar*	SMAX5B_012257
tr|A0A3B3THM5|A0A3B3THM5_9TELE	17.86	Histone H4	*Cyprinodon variegatus*	NA
tr|G3KG82|G3KG82_MISMI	3.50	Warm-temperature-acclimation-associated 65-kDa protein	*Austrofundulus limnaeus*	WAP65-1
tr|A0A060XVS1|A0A060XVS1_ONCMY	5.80	Uncharacterized protein	*Scophthalmus maximus*	GSONMT00038488001
tr|A0A1A7XBL5|A0A1A7XBL5_9TELE	3.64	WD repeat domain 1	*Poecilia latipinna*	WDR1
tr|A0A0P7UMM5|A0A0P7UMM5_SCLFO	7.98	Actin, cytoplasmic 2-like	*Misgurnus mizolepis*	Z043_118570
tr|A0A0F8APN0|A0A0F8APN0_LARCR	2.69	Alpha-1-antitrypsin	*Oncorhynchus mykiss*	EH28_09619
tr|A0A3B3HRV0|A0A3B3HRV0_ORYLA	6.08	Ig-like domain-containing protein	*Iconisemion striatum*	NA
tr|A0A3Q2GKU3|A0A3Q2GKU3_CYPVA	1.18	Anaphase-promoting complex subunit 5	*Scleropages formosus*	NA
tr|A0A4U5UXM5|A0A4U5UXM5_COLLU	0.85	Gelsolin Actin-depolymerizing factor	*Larimichthys crocea*	D9C73_013700
tr|Q5SEP6|Q5SEP6_GRASX	7.27	Histone H3 (Fragment)	*Oryzias latipes*	NA
sp|Q6PHG2|HEMO_DANRE	2.68	Hemopexin	*Cyprinodon variegatus*	hpx
tr|A0A1A7ZD11|A0A1A7ZD11_NOTFU	1.75	Abelson helper integration site 1	*Collichthys lucidus*	AHI1
tr|A0A146QB50|A0A146QB50_FUNHE	7.86	Histone H2A type 2-A (Fragment)	*Grammistes sexlineatus*	NA
tr|A6QL59|A6QL59_DANRE	8.66	Histone H2A	*Danio rerio*	hist1h2a6
tr|A0A0R4IQ11|A0A0R4IQ11_DANRE	1.25	Scinderin-like a	*Nothobranchius furzeri*	scinla
tr|A0A437C175|A0A437C175_ORYJA	2.18	Uncharacterized protein	*Fundulus heteroclitus*	OJAV_G00232420
			Danio rerio	
BAND 5			*Danio rerio*	
tr|A0A498MKB6|A0A498MKB6_LABRO	16.47	L-lactate dehydrogenase	*Oryzias javanicus*	ROHU_026592
tr|A0A1A7Z665|A0A1A7Z665_9TELE	11.68	L-lactate dehydrogenase		LDHB
tr|A0A3Q2XWT5|A0A3Q2XWT5_HIPCM	7.69	L-lactate dehydrogenase		NA
tr|I3IZU4|I3IZU4_ORENI	7.49	L-lactate dehydrogenase	*Labeo rohita*	LOC100694281
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	4.82	Putative histone H2B type 2-E-like	*Iconisemion striatum*	SMAX5B_012257
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	14.81	Histone H3	*NA*	NA
tr|A0A3B3THM5|A0A3B3THM5_9TELE	17.86	Histone H4	*Oreochromis niloticus*	NA
tr|A0A2I4CE20|A0A2I4CE20_9TELE	9.20	Histone H3-like	*Scophthalmus maximus*	LOC106527799
tr|A0A3B1J9L7|A0A3B1J9L7_ASTMX	15.38	Hemoglobin subunit alpha	*Cyprinodon variegatus*	NA
tr|A6QL59|A6QL59_DANRE	8.66	Histone H2A	*Poecilia latipinna*	hist1h2a6
tr|A0A146QB50|A0A146QB50_FUNHE	7.86	Histone H2A type 2-A (Fragment)	*Austrofundulus limnaeus*	NA
tr|A0A3B3HRV0|A0A3B3HRV0_ORYLA	6.08	Ig-like domain-containing protein	*Astyanax mexicanus*	NA
tr|A0A3Q2GKU3|A0A3Q2GKU3_CYPVA	1.18	Anaphase-promoting complex subunit 5	*Danio rerio*	NA
tr|Q5SEP6|Q5SEP6_GRASX	7.27	Histone H3 (Fragment)	*Fundulus heteroclitus*	NA

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