Kuroshio Science 9-1, 65-79, 2015

Sonic-muscle proteomics of ophidiid, glaucosomatid, pempherid and terapontid reveals the physiological and phylogenetic constrains to the sound producing structures

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Abstract

Among the soniferous percoids, glaucosomatid, pempherid, and terapontid share a fenestrum-like structure in the front part of their swim bladder that might play role in sound production. Previous molecular study suggested that the first two groups form a clade (monophyletic group). It is of great interest to compare the proteomic features of these groups with that of other soniferous fish (e.g., ophiidiform) so that the effects of functional structure and phylogeny to the proteomic characteristics of the sonic muscle (SM) can be compared. A species was selected for each of these four groups and their proteomics were analyzed. This study revealed the protein expression patterns of the sonic muscles in the ophiidiform species was more similar to that of the pempherid species. The proteins contribute to the close relationship between these two groups was discussed. A total of 484 protein spots were found in these four species, and 8 proteins were present in all SMs and differentially expressed among SMs of four species. Among these 8 proteins in SMs, 3 of them were present only in SMs and identified as: flotillin-1, HBS1-like protein, and ras-related protein ralB-B. Their functions which may be related to the specific role of the sonic muscle were discussed. This study combined 2D-GE and multivariate analysis provides a data set to review the applicability of proteomic approach on functions of the sonic muscle and phylogenetics in the soniferous fishes.

Keywords: proteomics; sonic muscle; multivariate analysis; two-dimensional gel electrophoresis (2D-GE); phylogeny

Introduction

Over 40 to 50 families of bony fishes produce sounds for social interaction or reproduction (Vester et al., 2004). Stridulation of bony structures, drumming of swim bladder by the sonic muscles, and hydrodynamic movement of water through structure (e.g., gill opening) are the three main ways for fishes to produce these sounds. When there are no sonic muscles, the swim bladder may serve to amplify the stridulatory sounds. However, most soniferous fishes possess sonic muscles which are either (1) originate from the cranium or other bones associated with the anterior vertebrae and insert to the swim bladder or solely lie on the inner body wall (i.e., the extrinsic sonic muscles), or (2) solely associated with the swim bladder (i.e., the intrinsic sonic muscles). The former is more common of the two. The sonic muscles contract and relax at high speed forcing the swim bladder to pulsate and produce the sound (Ladich and Fine, 2008). As sonic muscles are the crucial structure for generating the sounds, many studies have focused on their characteristics (including histological, chemical and proteomic ones).Take *Micropogonias furnieri*, a sciaenid species, as an example, the fibers of the sonic

Received 31 August 2015; revised and accepted 9 September 2015.

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muscles, which were smaller than the white myogomal and pectoral fibers, showed homogenous size and distribution and had features common to white fibres (i.e., negative to SDH and lipids, weakly positive to PAS and m-ATPase following acid pre-incubation, and positive to m-ATPase at alkaline pre-incubation; Devincenti et al. 2012). Concentrations of glycogen, fat, and water in toadfish fast sonic muscle were higher than the corresponding concentrations in white body muscle (Fine et al. 1986); these differences was considered relevant to adaptation of the sonic muscle for fast oxidative glycolytic fiber type.

Because fish sonic muscle is considered the fastest vertebrate muscle, proteins related to its metabolism are expected to be different from other muscle types. One of the physiological adaptations is that the rates of cross-bridge dissociation and Ca^{2+} transport are fast in this muscle type (Chiu et al. 2013). Parvalbumins are Ca^{2+}-binding proteins that help muscle relaxation in vertebrates, percentages of their isoforms were found different between the sonic and white muscles (Chiu et al. 2013).

Being the fastest muscles, the rate of the contraction and relaxation (i.e., cycle time) in sonic muscles may reach 400 Hz without fatigue. This rate determines the fundamental frequency of the sounds which they emitted (e.g., the oyster toadfish contracts its intrinsic sonic muscles at 200 Hz to produce a courtship call with that fundamental frequency; Fine et al., 2001). However, slow sonic muscles have been discovered in ophidiiforms (e. g., carapids); these muscles tetanize around 10 Hz (Parmentier et al. 2006). The sounds of ophidiiforms are produced not by the contraction of the sonic muscles, but emitted during the recoiling of the stretched anterior swimbladder; the recoiling apparatus involves with either a swimbladder fenestra or a rocker bone associated with the swim bladder. The anterior sonic muscles of pearl-perch, *Glaucosoma buergeri* were found contracting at a cycle time of around 212 Hz and can be treated as fast sonic muscles (Mok et al. 2011). However, these forced movement did not generate significant sound, while the main part of the sound was generated by the recoiling of the anterior swimbladder − a mechanism similar to, but not identical to that of the carapids; it also has a well-developed fenestra but with a single tendon + smooth muscle recoiling structure to recoil the anterior part of the swim bladder; Mok et al. 2011).

Beside the pearl-perch, pempherids and terapontids were found to have a similar recoiling system (Mok, unpublished data; Hsueh 2013). Similarity in the sound-producing apparatus (particular between the pearl-perch and pempherid) suggests that these three families may have been derived from a common ancestor. Molecular evidences, however, only support the sister-group relationship between pearl-perch and pempherid, while terapontid is a more remote group (Jiang, 2010; Ricardo et al., 2013).

The above-mentioned structural variations in the sound-producing system in fishes can be summarized to an evolutionary trend with the following stages: slow extrinsic mucle + recoiling apparatus (sound produced by the latter structure; e. g., carapids), fast extrinsic muscle + recoiling apparatus (sound mainly produced by the latter structure; e.g., glaucosomatid), fast extrinsic muscle only (e.g., berciforms, e g., holocentrids and perciforms, e. g., most sciaenids), fast intrinsic muscle only (e.g., toadfish, some sciaenids).

Proteomics is a large-scale analysis of proteins in an organismic system which allows us to understand the proteins' composition, structure and function. However, it has also been used for phylogeny-based comparative analysis. For example, venome proteome variation in a clade of rattlesnakes was analyzed to construct the phylogeny of the clade members (Gibbs et al. 2013). For the functional aspect of sonic muscle, proteomic analysis found the expressions of creatine kinase, fast muscle myosin heavy chain, isocitrate dehydrogenase, and cytochrome P450 monooxygenase were higher in the hypertrophic extrinsic sonic muscle of the big-snout croaker, *Johnius macrorhynus* in the spawning season suggesting the importance of these proteins for the muscles' fast performance (Lin et al. 2011). As mentioned above, functional specificity of the sonic muscles varies among soniferous groups. Variations in the proteomic feature in the sonic muscles with rate difference may also show genealogical basis rather than solely determined by biochemical or physiological functions. To test this theoretical idea, proteome of the sonic muscles in ophidiiforms (with slower sonic muscle), glaucosomatid, pempherids, and terapontids (with fast sonic muscle) are compared. Expected results include: (1) Proteomic features in the taxa with fast sonic muscles should be more similar (including the second to fourth taxa); (2) proteomic features in glaucosomatid and pempherid which are sister taxa should be more similar to each other than to the other two taxa compared. Results of this study should also provide additional knowledge about the proteomic differences between slow sonic muscle, fast sonic muscle and white muscles.

Materials and Methods

Sample collection

All of the animal care and use procedures were approved by the Institutional Animal Care and Use Committee of National Sun Yat-sen University before the study began. **Figure 1** describes the experimental design of sample preparation. All the specimens were obtained from the Southwestern waters of Taiwan. Live glaucosomatid (*Glaucosoma buergeri*) and terapontid (*Terapon jabua*) were caught by fishermen and bought from the seafood wholesaler

in Kaohsiung City. The ophidiids (*Hoplobrotula armata*) were bought in ice condition from seafood wholesaler in Tongkang, Pingtung. Because the ophidiid specimens were not possible to keep alive after they were caught, the fishermen were asked to put the sample in ice immediately when they caught the specimens. The pempherid (*Pempheris oualensis*) specimens were obtained by hooked and line at Sizihwan Bay, Kaohsiung City, Taiwan. Sonic muscle (SM) and white muscle (WM) were sampled from these specimens and were directly used or frozen at -70℃ for later proteomic analysis. The swim bladders and their associated sonic muscles in the experimental animals described in **Figure 2.**

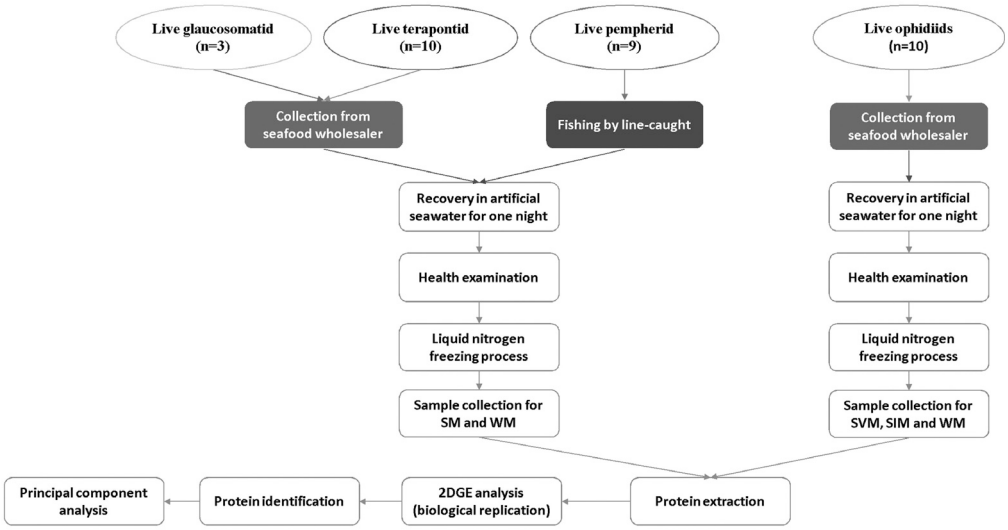

Fig.1. The experimental design of fish sample collection and preparation.

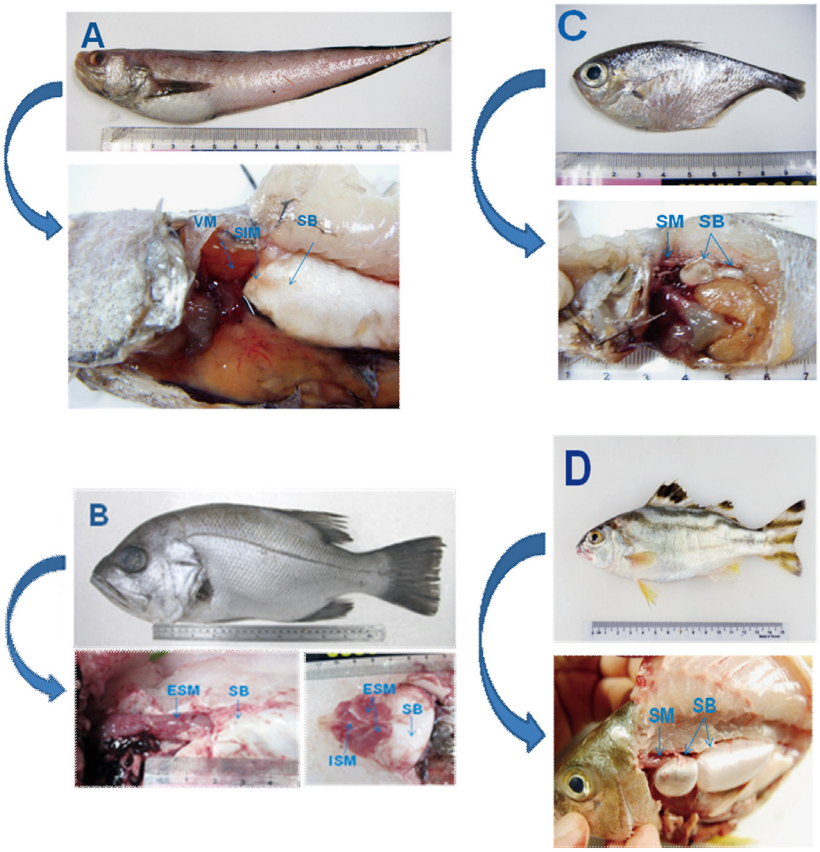

Fig.2. Swim bladder and its associated sonic muscle in (A) *H. Armata,* (B) *G. buergeri* (C) *P. Oualensis* (D) *T. Jarbua.* SM (sonic muscle), SB (swim bladder), VM (ventral sonic muscle), ISM (intermediate sonic muscle), ESM (eksternal sonic muscle), SIM (internal sonic muscle).

Preparation of protein extraction

Muscle proteins were extracted according to the method described by Chiu et al. (2007) and Lin et al. (2011). SM and WM were sampled from 3 glaucosomatid specimens, Nine pempherid specimens and 10 terapontid specimens. WM and ventral sonic muscle and intermediate sonic muscles from 10 ophidiid specimens were sampled. Protein concentrations were measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Two-dimensional gel electrophoresis (2D-GE)

18-cm Immobiline Dry-Strip pH 3-10 (GE Healthcare Bio-Sciences AB, UK) was used in the first dimension electrophoresis (IEF) analysis. The strip containing muscle protein was actively rehydrated for 14 hours, at 50V, 20℃ on the electrophoretic apparatus, then directly continued with IEF steps begun at 500 V for 3 hours, 1,000 V for 3 hours, then gradient to 10,000 V for 3 hour, and finally 10,000 V until 60,000 V-hours was reached.

The focused strips were equilibrated for 20 minutes in SDS equilibration buffer solution (6 M urea, 20% glycerol, 2% SDS, 0.01 % bromophenol blue, and 50 mM Tris-HCl at pH 8.8) with 30 mM DTT. Second equilibration step was then performed using equilibration solution with 135 mM IAA replacing the DDT mixture solution. The second dimension separation was performed with 12.5% SDS-PAGE gel at 25 mA per gel running, constant current on a vertical electrophoresis unit until the dye front was approximately 1 mm from the bottom of the gel.

Technical replicates of 2D-GE gels were carried out three times in each sample.

Gel staining

After electrophoresis had been completed, the gels were then taken out from the glass and stained using a modified colloidal Coomassie G-250 staining method (Blue Silver) (Candiano et al. 2004).

Gel scanning and image analysis

The stained 2G-DE gels were visualized and digitalized by ImageScanner^{TM} (Amersham Bioscience). The scanned TIFF images were analyzed using PDQuest software. Spots were automatically detected and visually checked for undetected or incorrectly detected spots. The protein spots detected in each experimental gel were matched to their corresponding spots in a digitized reference gel. Intensity levels were normalized between gels by dividing the spot intensity by the total intensity of all the spots in the gel. The differences in spot intensities were analyzed by the ANOVA followed by Turky test (assuming normal distributions and equal variance). A value of $p < 0.05$ was required for statistically significant differences. In addition, after background subtraction, the criteria for spot "absent" or "present" was based on the selected spots which had lowest intensity and minimal area set in the master gel generated by PQDuest software. If the spot intensity and area was lower than the criteria, it would not be detected and be considered as "absence"; by contrast, it would be detected and considered as "presence".

Protein identification

Spots of interests from the SM gels were chosen to be identified. A total of 8 spots were excised from 2G-DE gels and washed, distained, dehydrated, and in-gel digested with trypsin (Promega, Madison, WI, USA) at 37℃ overnight. The tryptic peptides were re-extracted twice in 10 μL of a 5% formic acid and 50% acetonitrile (ACN) solution that was treated for 15 minutes in a water-bath sonicator. The extracted peptide solution was concentrated by vacuum centrifuge, and dissolved in 2 μL of 5% formic acid. The peptides were mixing with matrix solution [50% ACN, 0.5% trifluoroacetic acid (TFA) saturated with a-cyano-4-hydroxycinnamic acid (CHCA)] and dried on the sample plate. The mass spectra of the digested peptides from each spot were analyzed using a MALDI-TOF/TOF (Bruker Autoflex III) mass spectrometer. The proteins were identified by MS/MS fragmentation spectra searching within the Swiss-Prot or NCBInr databases, using the search program MASCOT (http: //www. matrixscience. com). The searches were taxonomically restricted to Actinopterygii (ray-finned fishes). The mass search parameters were set as follows: peptide mass tolerance, 1Da ; MS/MS tolerance, 1Da; peptide charge, + 1,+ 2, and + 3; data format, sequence (DTA); instrument, MALDI-TOF-TOF; missed cleavage, 2; consideration for variable modifications such as carbamidomethyl, deamidated, and oxidation.

Data analysis

Cluster analysis

Specimens of the studied soniferous species were clustered using their sonic muscle proteomic data. Intersections of the protein-spots intensities among the four species, analyzed by Boolean analysis (with minimal 2-fold difference), were compared using *Student*'s *t*-test. Un-weighted pair-group method using arithmetic average (UPGMA) was selected for measuring group distance (Romesburg, 1989).

Single linkage clustering method was used to group the operational taxonomic units (OTUs).

Principal Component Analysis (PCA)

Principle component analysis (PCA) was conducted on all normalized data of the intensities for the sonic-muscle protein spots from the 2D-GE gels. Three gels, whose spot numbers were at the most three maximum, from each species were selected for PCA analysis. PCA was performed using Minitab software version 13.1. The score plot of PC1 versus PC2 was examined for separation of clusters related to protein abundance. The loading biplot of PC1 and PC2 was examined to reveal (1) possible groupings of OTUs and (2) the informative spots (proteins) for showing the differences among the groups of OTUs.

Results

Inter-muscle differences in 2D-GE gel protein spots of *G. buergeri, P. oualensis, T. jabua*, and *H. armata.*

The 2D-GE gels of SM and WM of *T. jabua, G. buergeri, P. oualensis,* and *H. armata* are shown in (**Supplementary Figures 1-4**).

In *T. jarbua*, a total of 42 protein spots were significantly different ($p < 0.05$) between SM and WM, and 30 SM spots were up-regulated and 12 SM spots were down-regulated. Among these differentially expressed protein spots, 17 were present only in SM, and 5 were present only in WM (**Table 1**).

In *G. buergeri,* a total of 45 protein spots were significantly different ($p < 0.05$) between SM and WM, and 37 SM spots were up-regulated and 8 SM spots were down-regulated. Among these differentially expressed protein spots, 26 were present only in SM, and 5 were present only in WM (**Table 1**).

In *P. oualensis,* a total of 51 protein spots were significantly different ($p < 0.05$) between SM and WM, and 35 SM spots were up-regulated and 16 SM spots were down-regulated. Among these differentially expressed protein spots, 16 were present only in SM, and 11 were present only in WM (**Table 1**).

In *H. armata*, a total of 53 protein spots were significantly different ($p < 0.05$) between WM and SM, and 32 SM spots were up-regulated and 14 SM spots were down-regulated. Among these differentially expressed protein spots, 28 were present only in SM, and 8 were present only in WM (**Table 1**).

Table 1. Numbers of protein spots of sonic muscle (SM) relative to white muscle (WM) that were significantly different ($p < 0.05$) from the four species.

	Terapon jarbua	*Glaucosoma buergeri*	*Pempheris oualensis*	*Hoplobrotula armata*
Number of protein spot differentially expressed	42	45	51	53
SM up-regulated	30 (17*)	11 (26*)	19 (16*)	4 (28*)
SM down-regulated	12 (5**)	3 (5**)	5 (24**)	6 (8**)

(*) = number of spots only present in SM; (**) = number of spots only present in WM

Inter-specific differences in 2D-GE gel protein spots of sonic muscles of *G. buergeri, P. oualensis, T. jabua,* and *H. armata*

Expressions of the protein spots showed dramatically different patterns among these four species. Intersection of protein spots among the four species by Boolean analysis (minimal 2-fold change) revealed that 143 spots were significantly different (*Student's* *t*-test, $p < 0.05$) between *G. buergeri* and *P. oualensis.* 134 spots were significantly different between *G. buergeri* and *T. jabua,* 109 spots were significantly different between *G. buergeri* and *H. armata*. 107 spots were significantly different between and *P. oualensis* and *T. jabua,* 86 spots were significantly different between *P. oualensis* and *H. armata*. 99 spots were significantly different between *T. jabua* and *H. armata* (**Table 2**).

The intersection of protein-spot intensities from the four fish species shows that *Hoplobrotula* and *Pempheris* are more closely similar in their protein composition when clustered by single linkage method (**Fig. 3**). PCA result also reveals that *Hoplobrotula* and *Pempheris* were grouped together based on their protein composition, although one *Hoplobrotula* sample was excluded in PCA scores plot (**Fig. 4**).

Table 2. Numbers of protein spot intersected between *Terapon jarbua, Glaucosoma buergeri, Pempheris oualensis,* and *Hoplobrotula armata* analyzed by Boolean analysis (Quantitative 2-fold and *Student's* *t*-test p = 0.05). Numbers in parentheses represent the spots not significantly differred in intensity.

Species	*Terapon jarbua*	*Glaucosoma buergeri*	*Pempheris oualensis*	*Hoplobrotula armata*
Terapon jarbua		134 (161)	107 (188)	99 (196)
Glaucosoma buergeri			143 (152)	109 (186)
Pempheris oualensis				86 (209)
Hoplobrotula armata				

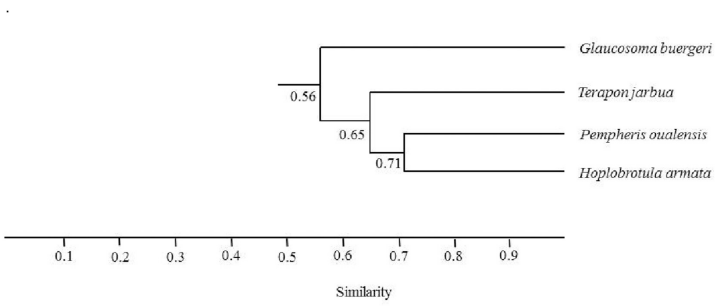

Fig.3. Cluster dendrogram of similarity in intensity of protein spots of the sonic muscle in *Hoplobrotula armata, Terapon jarbua, Pempheris oualensis,* and *Glaucosoma buergeri.* Similarity measure: Jaccard coefficient; clustering method: unweighted pair-group method using arithmetic average.

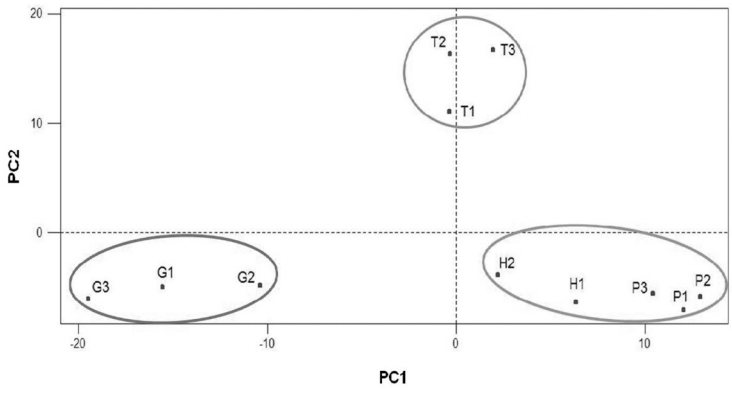

Fig.4. Principal component analysis (PCA) of protein expression (484 spots, normalized) in sonic muscles of the four fish species (G = *G. buergeri,* T = *T. jarbua,* H = *H. armata,* P = *P. oualensis*).

To search what proteins consistently expressed in the sonic muscles of these four species, we selected the proteins expressed the SMs in these four species but differentially expressed among them. A total of 484 spots were found in master gel and 8 spots exhibiting significant difference in intensity among the SM of these four species. The number codes of these 8 protein spots were number 110, 205, 214, 303, 307, 316, 419 and 420 (**Fig. 5**).

Among these 8 protein spots, all spots were up-regulated in SM compared to WM, and 3 spots were only present in the SMs of all the four species (i.e., absent from WMs; spots 214, 307, and 316) in comparing with the WM. These proteins were identified as ras-related protein ralB-B (spot 214), flotillin-1 (spot 307), HBS1-like protein (spot 316), ATP synthase subunit beta (spot 110), nascent polypeptide-associated complex subunit alpha-2 (spot 205), troponin T (spot 303),

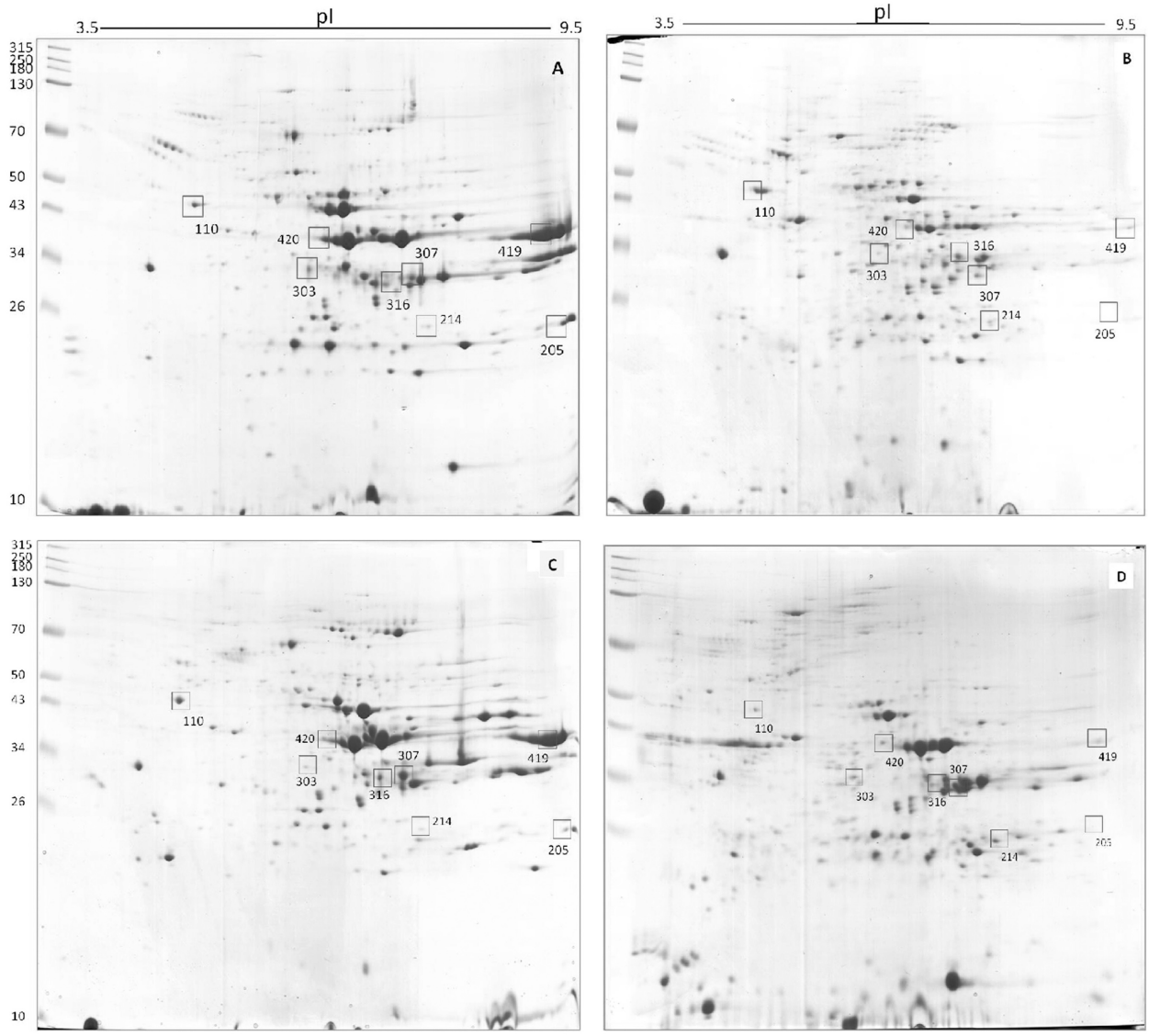

Fig.5. The 2D-GE maps of the sonic muscle in four fish species. (A) *Glaucosoma buergeri,* (B) *Pempheris oualensis,* (C) *Terapon jarbua,* and (D) *Hoplobrotula armata*

actin-alpha skeletal muscle (spot 419), and creatine kinase M-type (spot 420) (**Table 3**).

The contribution of these protein involved in the separation of SMs was listed in **Table 4**. According to PC eigenvectors of identified proteins in PCA results, ATP synthase subunit beta, actin-alpha skeletal muscle, HBS1-like protein and nascent polypeptide-associated complex subunit alpha-2 were positively associated with PC 1, whereas flotillin-1 and creatine kinase M-type were negatively associated with this component. On the other hand, all proteins, except actin-alpha skeletal muscle, were positively correlated with PCs 2. Based on the quadrant position, troponin T, HBS1-like protein and actin-alpha skeletal muscle

Table 3. Summary of identified muscle protein and the presence in the sonic muscle (SM) and the white muscle (WM)

Spot number	Accession number	Score	Peptides matched	Coverage (%)	Protein name	Presence SM/WM
303	AAK92231.1	20	29	20	Troponin T	SM/WM
110	P06576	27	14	16	ATP synthase subunit beta, mitochondrial	SM/WM
419	P02568	29	10	22	Actin, alpha skeletal muscle	SM/WM
307	O75955	20	15	9	Flotillin-1	SM
316	Q9Y450	22	15	13	HBS1-like protein	SM
205	P70670	22	11	13	Nascent polypeptide-associated complex subunit alpha-2	SM/WM
214	B5X6D9	20	32	18	Ras-related protein ralB-B	SM
420	P06732	32	24	22	Creatine kinase M-type	SM/WM

Table 4. Spot identification number, protein name and the eigenvectors of the PC1 and PC2.

Protein Name	Spot No.	PC 1	PC 2
1. Troponin T	303	-0.041	0.039
2. ATP synthase subunit beta, mitocondrial	110	0.025	0.067
3. Actin, alpha skeletal muscle	419	0.050	-0.010
4. Flotillin-1	307	-0.070	0.040
8. HBS1-like protein	316	0.000	0.089
9. Nascent polypeptide-associated complex subunit alpha-2	205	0.007	0.101
10. Ras-related protein ralB-B	214	0.003	0.005
11. Creatine kinase	420	-0.002	0.029

Table 5. Coefficient of similarity between *Terapon jarbua, Glaucosoma buergeri, Pempheris oualensis,* and *Hoplobrotula armata* by Boolean analysis (Quantitative 2-fold + *Student*'s *t*-test p = 0.05).

Species	*Terapon jarbua*	*Glaucosoma buergeri*	*Pempheris oualensis*	*Hoplobrotula armata*
Terapon jarbua		0.55	0.64	0.66
Glaucosoma buergeri			0.52	0.63
Pempheris oualensis				0.71
Hoplobrotula armata				

contributed to the variations of *G. buergeri, T. jarbua* and (*P. oualensis, H. armata*), respectively. The results of cluster analysis suggests the following groups for the sonic muscles' proteomic features: (Glaucosoma (Terapon (Hoplobrotula, Pempheris))) (**Figs. 3 and 4**).

Coefficient of similarity based on the protein spot intensities between *Terapon jarbua, Glaucosoma buergeri, Pempheris oualensis,* and *Hoplobrotula armata* by Boolean analysis also showed that the similarity was highest between *Pempheris oualensis*, and *Hoplobrotula armata* (0.71), and lowest between *Terapon jarbua* and *Glaucosoma buergeri* (0.55) (**Table 5**). These results also support the assumtion described above based on **Table 4, Figures 3 and 4.**

Discussion

Proteomic analyses of sonic muscle and white muscle

The SMs of the four species in this study were considered as a red/pink muscle type. Fine et al. (1986) suggested that SMs as described by color are typically red. Red muscles are rich in glycogen, using lipid as a fuel and may have a lipid content of up to 30 % wet mass more than that in the WM (Connaughton 1997). Similarly, Parmentier et al. (2003) found that the fibers of the SM contain higher amount of glycogen and mitochondria. On the other hand, Fine et al. (1993) revealed that the SM fibers and myofibrils are thinner than that in the WM, but the sarcoplasmic reticulum developed substantially and the calcium ionic content is several times higher in the SM (Hamoir et al. 1980; Gillis 1985). In association with these differences, difference in expression of certain proteins can be expected. The 2D-GE proteomic analysis method used in this study provided good separation of protein expressions in the sonic and WMs of *G. buergeri, P. oualensis, T. jabua*, and *H. armata* with high resolution. More proteins were up-regulated in the sonic muscles of the first three species than their white muscles suggesting that these fast SM have more functional needs for its contraction. Interestingly, fewer SM proteins in *H. armata* were up-regulated than its WM; its sonic muscle is considered as slower muscle (see above).

Proteins of SM may be classified as muscle contractile apparatus protein, energy metabolism protein, and miscellaneous protein (Lin et al. 2011), and the functions of identified proteins were discussed as follows:

Muscle contractile apparatus protein:

Actin, alpha skeletal muscle (spot 419) is the most abundant protein in striated muscle. This protein has three different kinds of isoforms-alpha, beta, and gamma. In this study, *actin, alpha skeletal muscle* was found with higher expression in the SM than in WM and it is probably due to its higher motility in the sonic-muscle cells which supports their rapid contracting activity. Nahirney et al. (2006) reveal that actin leptomeres as myofibril assembly/disassembly intermediates in SM fibers.

Troponin T (spot 301) is important to regulate striated muscle contraction in response to fluctuations in intracellular calcium concentration. The speed of the transport of calcium ions in the SMs is substantially larger than in other muscles of vertebrates (Fine et al. 1993; Rome et al. 1996). As mentioned by Fine et al. (1990), SM needs rapid flows of metabolites and calcium than the WM and a sufficient energetic inflow is supplied by their large amount of mitochondria (Fine et al., 1993). Hamoir et al. (1980) and Gillis (1985) also mentioned that the sarcoplasmic reticulum in the SM is developed substantially better than in the WMs. In addition, the calcium ionic content in the sarcoplasm of the SMs is several times higher than in the WMs.

Nascent polypeptide-associated complex subunit alpha-2 (spot 207) required for myofibril organization which is composed of highly organized repetitive structures called sarcomeres, the basic contractile unit of striated muscle (Li et al. 2009). This protein involves in transcription regulation and mitochondrial protein import, and also prevents inappropriate targeting of non-secretory polypeptides to the endoplasmic

reticulum (ER). It is expressed specifically in testis and skeletal muscle.

Energy metabolism protein:

ATP synthase subunit beta (spot 110), which produces ATP from ADP, is found with a higher expression in the SM comparing with the WM. This protein is important to produce ATP from ADP as a intracellular energy transfer.

Creatine kinase M-type (spot 410) is also up-regulated in the SM. This protein plays a central role in energy transduction as the SM need high energy for their contraction. Lin et al (2011) revealed that the creatine kinase expression was higher in the hypertrophic muscle of the big-snout croaker *Johnius macrorynus*. Ou-Yang (2010) found several types of creatine kinase in the slow SM of ophiidid fish, i.e., creatine kinase-2, creatine kinase muscle isoform 1, muscle creatine kinase b, and creatine kinase M2-CK, suggesting that these proteins are important in SM energy metabolic processes.

Miscellaneous proteins:

Three spots (spots 214, 307, and 316), which were absent in WMs but shared by all sonic muscles of the studied species, may be treated as miscellaneous proteins. They were identified as ras-related protein ral-B, flotillin-1, and HBS1-like protein, respectively. Ras-related protein ral-B is involved in gene expression, cell migration, cell proliferation, oncogenic transformation and membrane trafficking. Frotillin-1 may act as a scaffolding protein within caveolar membranes, and it is involved in formation of caveolae or caveolae-like vesicles and highly expressed in skeletal muscle. HBS1-like protein may act as a GTP binding. However, the physiological functions of these proteins in fish SMs were still unclear.

Clustering analyses of sonic muscle from *H. armata, G. buergeri, P. oualensis,* and *T. jabua*

Different protein expression in the SMs among *G. buergeri, P. oualensis, T. jabua,* and *H. armata* might be due to different functional needs as their sound-producing apparatuses differ to a certain degree (e.g., Mok et al. 2011; see above). The highest number of protein spots with significantly difference in contents appeared between *G. buergeri* and *P. oualensis* and it suggests that their sonic muscles might be more dissimilar in their physiological performance. This finding, however, contradicts with their close phylogenetic affinity and structural similarity in their sound producing apparatus (i.e., swim bladder morphology; Jiang 2010). The SM of *G. buergeri* is obviously deeper (deep red) in their red coloration than the other three species studied (Mok, unpublished data). The deeper red coloration in *G. buergeri* might be due to the higher formation of blood vessels and capillaries. Kasumyan (2008) suggested that the large vascularization of the SM contributes to intensive red coloration and the previous investigation by Jones and Marshall (1953) has revealed that SM in genus *Prionotus* are composed of 'red striated fibers, indicating presence of myoglobin. While the SM colors of *P. carolinus* and *P. evolans* are white, indicating absence of myoglobin (Evans 1968). Further, Parmentier et al (2003) find that myofibrils in SM formed the helicoidal organization so that a larger number of sarcomeres can be included, which, in turn, increases the number of actinmyosin bindings.

According to the clustering and PCA analyses based on the protein expressions in the SMs, *P. oualensis* and *H. armata* were grouped implying that these two species have certain similarity in their protein expression. This result disagrees with the predicted result if phylogeny plays more import role in determining protein pattern (see above). Jiang's study (2010) indicated that *P. oualensis* is the sister group of *G. buergeri*. More importantly, *H. armata* may not be as strong a sound producer as that of the *P. oualensis* and *G. buergeri,* because its muscles are consider slow. The reason behind the similarity demonstrated in *P. oualensis* and *H. armata* remains unclear. Kasumyan (2008) suggested that it is important to note that not all fishes within the same family have a similar protein composition in their SMs; these species should be more closely related to each other. However, on the basis the results of the present study, one inference is that proteomics, which is suitable for by physiological adaptation, may not be suitable for resolving phylogenetic relationship. The results of clustering suggest the following grouping for the sonic muscles' proteomic features: (*Glaucosoma* (*Terapon* (*Hoplobrotula, Pempheris*))) (**Fig. 3**). And troponin T, HBS1-like protein and actin-alpha skeletal muscle contributed to the variations of *G. buergeri, T. jarbua* and (*P. oualensis, H. armata*), thus these proteins are suggested to be protein markers for functional and phylogenetic classification in these soniferous species.

Further comparisons on the histological and acoustical characteristics among these four groups and detailed studies on the protein spots in the SMs are necessary to verify the reliability of this hypothetical grouping.

Conclusion

This study used the proteomic features in the taxa including ophidiid, glaucosomatid, pempherid and terapontid with fast sonic muscles to reveal the physiological and phylogenetic constrains, in addition, it should also provide additional knowledge about the proteomic differences between slow sonic muscle, fast sonic muscle and white muscles.

Acknowledgments

This study was supported by Grants 102-2313-B-022-002 from the National Science Council to KHC. The authors thank the National Cheng-Kung University Proteomics

Research Core Laboratory for assistance in mass spectrometry analyses. Data in this paper are from the first author's thesis submitted in partial fulfillment for the master degree at National Sun Yat-sen University.

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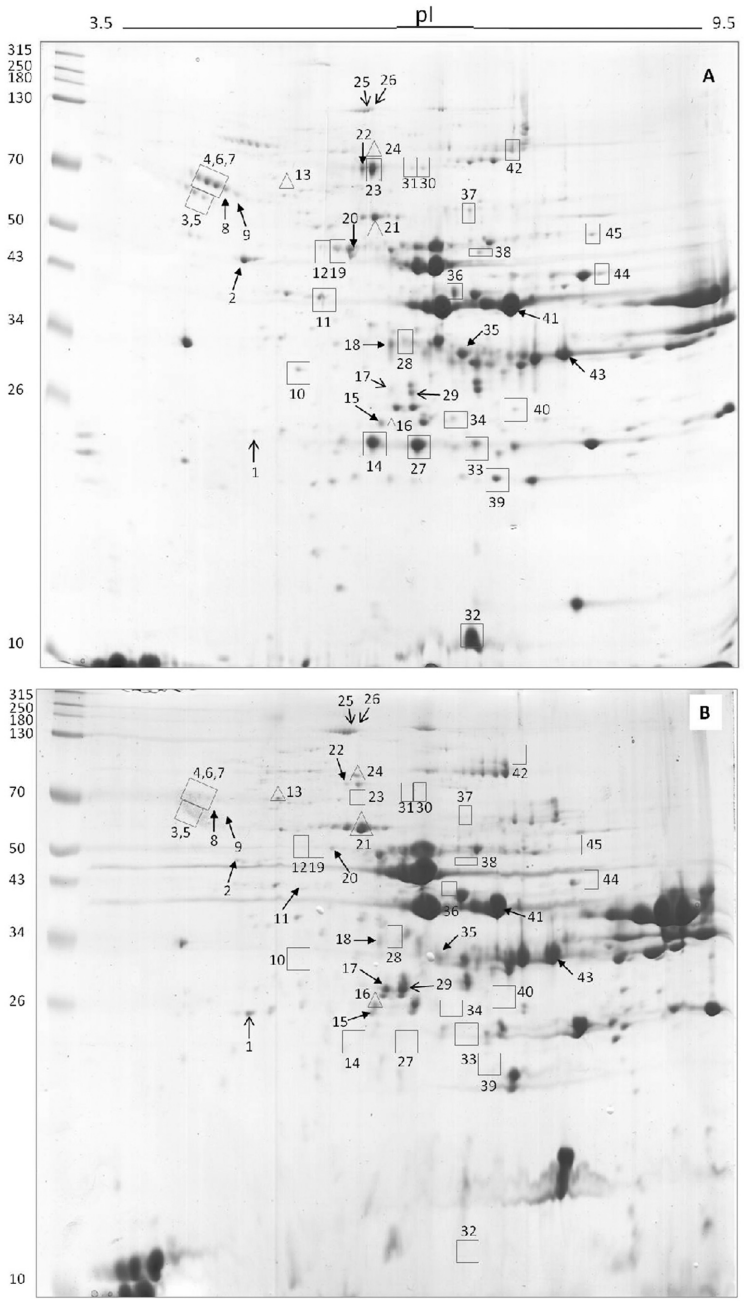
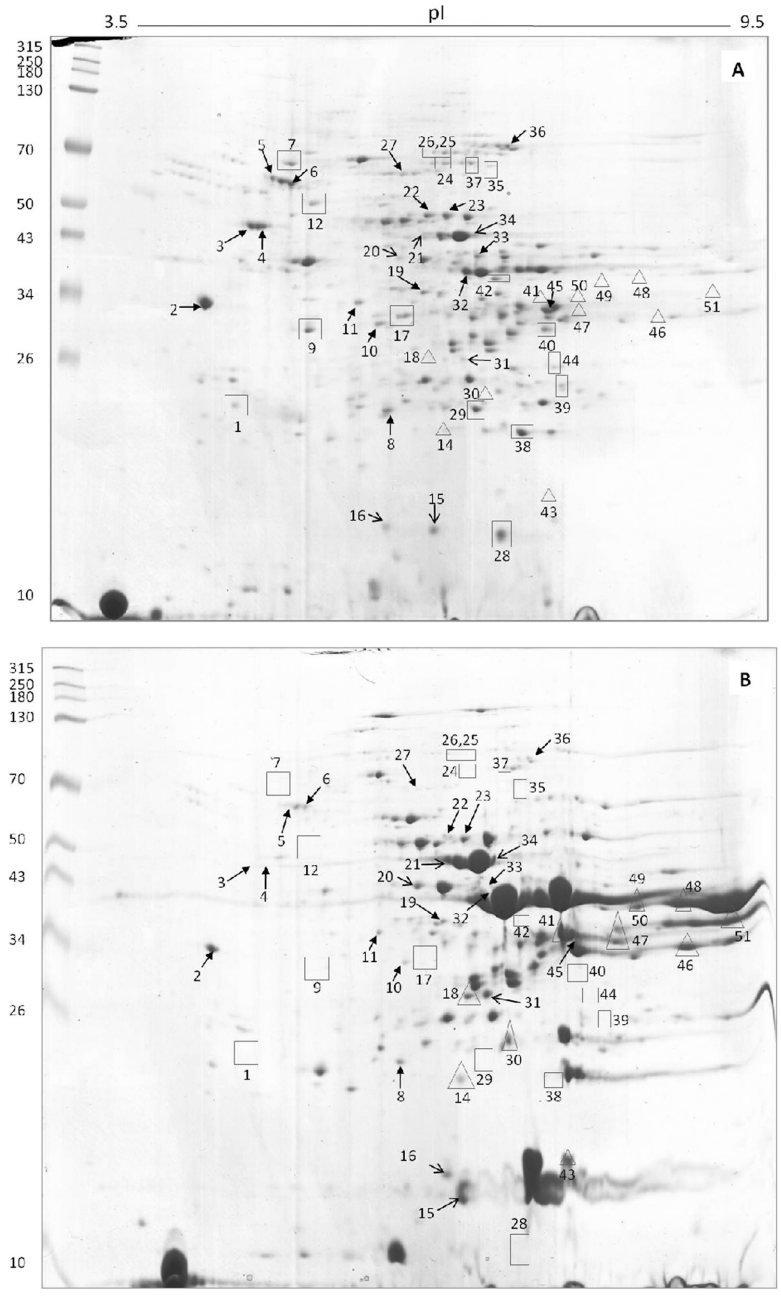
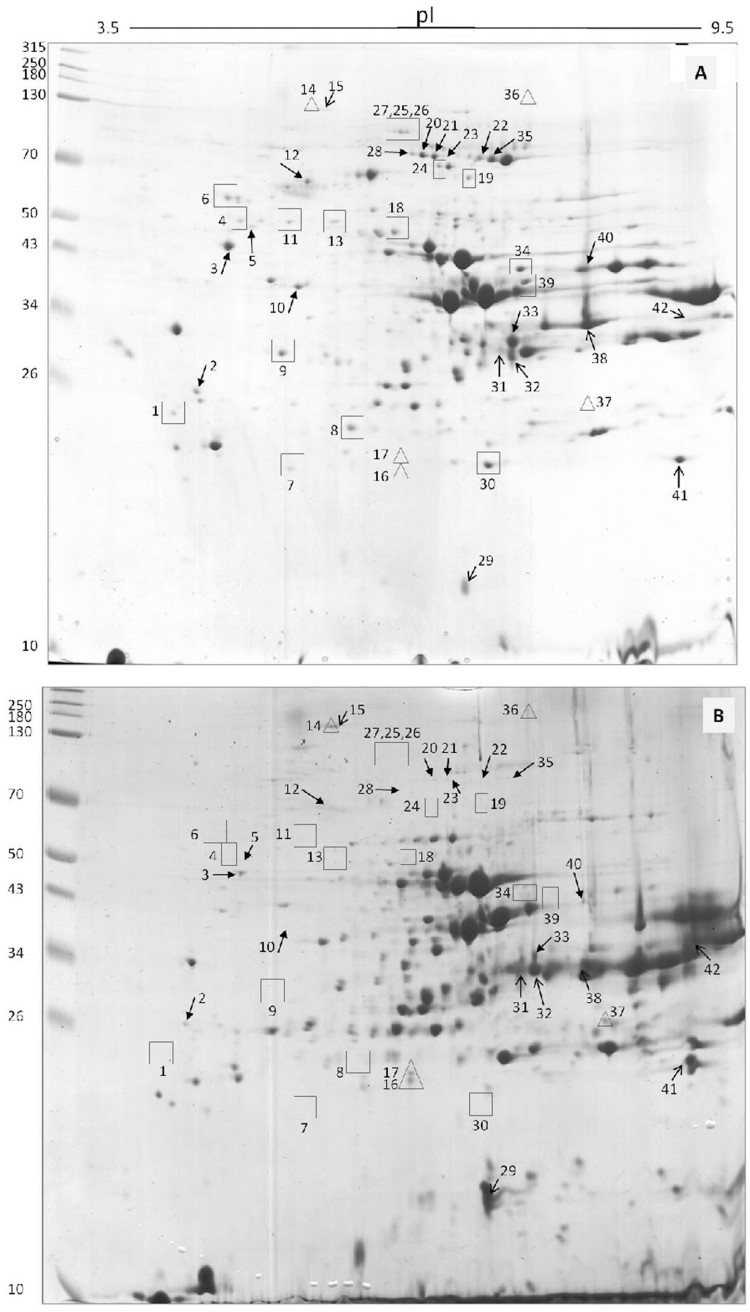


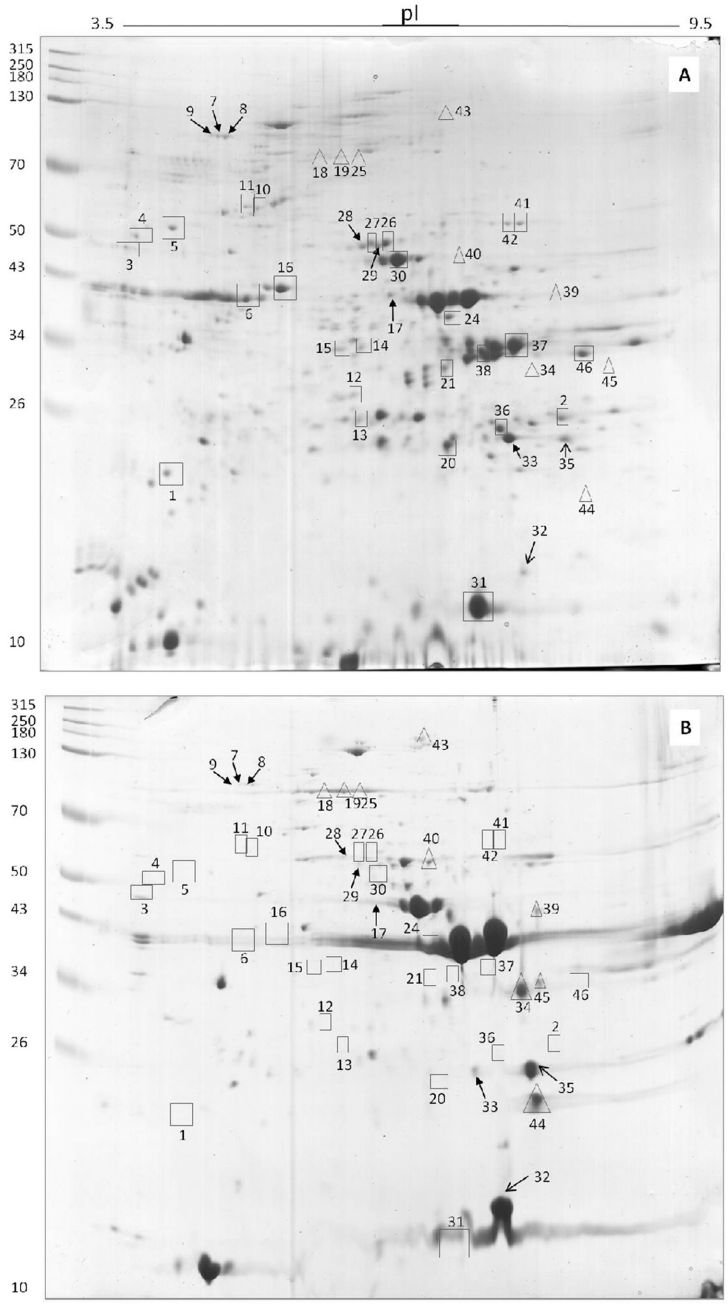
Supplenmentary Fig. 1. Two-dimensional gel of sonic muscle (A) and white muscle (B) of *Glaucosoma buergeri*. First dimension: immobilized Dry-Strip pH gradient 3-10, 18 cm; second dimension: vertical SDS polyacrilamide gel (12.5%). The protein spot intensities between white muscle and sonic muscle were significantly different ($p < 0.05$)



Supplenmentary Fig. 2. Two-dimensional gel of sonic muscle (A) and white muscle (B) of *Pempheris oualensis.* First dimension: immobilized Dry-Strip pH gradient 3-10, 18 cm; second dimension: vertical SDS polyacrilamide gel (12.5%). The protein spot intensities between white muscle and sonic muscle were significantly different ($p < 0.05$).



Supplenmentary Fig.3. Two-dimensional gel of sonic muscle (A) and white muscle (B) of *Terapon jarbua*. First dimension: immobilized Dry-Strip pH gradient 3-10, 18 cm; second dimension: vertical SDS polyacrilamide gel (12.5%). The protein spot intensities between white muscle and sonic muscle were significantly different ($p < 0.05$).



Supplenmentary Fig. 4. Two-dimensional gel of sonic muscle (A) and white muscle (B) of *Hoplobrotula armata.* First dimension: immobilized Dry-Strip pH gradient 3-10, 18 cm; second dimension: vertical SDS polyacrilamide gel (12.5%). The protein spot intensities between white muscle and sonic muscle were significantly different ($p < 0.05$).