International Journal of Biological Macromolecules 379 (2026) 153941

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Species-specific and processing-dependent fish allergen profile diversities: An integrated omics workflow for improving seafood safety, allergy diagnosis and management☆

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ARTICLE INFO

Dataset link: An Integrated Omics Workflow to Resolve Diverse Fish Allergen Profile for Improving Seafood Safety, Allergy Diagnosis and Management (Original data)

Keywords:
Allergenomics
Food allergy
Thermal processing
Parvalbumin
Food safety
Risk assessment
Quantitative proteomics

ABSTRACT

Seafood allergy is complex due to extensive species diversity, posing major challenges in food safety assessments, clinical diagnosis and dietary management. However, the absence of established workflows to resolve allergenomes limits correlations between allergen abundance, clinical sensitisation, and consumer risk. Mass spectrometry (MS)-based proteomics overcomes limitations of conventional immunoassay allergen detection by enabling unbiased protein identification and quantification, including allergen isoforms and low-abundance proteins within complex matrices. An integrated workflow combining immunological analyses, liquid chromatography-MS/MS proteomics, and bioinformatics was developed to characterise allergenomes across eight commonly consumed Asia-Pacific fish species. Comprehensive allergen profiles were established using *in silico* allergenicity predictions with AllerCatPro, combined with immunological validations using allergen-specific antibody and pooled patient sera. Across eight species, 529–1012 protein groups were identified, including all 11 fish muscle allergens, with pronounced interspecies differences in allergen composition and isoform distribution. The major fish pan-allergen parvalbumin was the most abundant allergen of most species and varied in abundance by up to 7-fold. Mackerel displayed a distinct low-parvalbumin profile with enriched metabolic allergens. Tissue heating induced a consistent shift toward enrichment of heat-stable and tissue-retained allergens, particularly parvalbumin, tropomyosin, and collagen. In contrast, heating of raw extracts generated more variable and species-specific retention of selected proteins, including heat-labile metabolic enzymes. *In silico* analysis predicted 14 proteins with strong allergenicity evidence for further validation. Fish allergenomes are species-specific and processing-dependent, positioning quantitative proteomics as a powerful platform for improved molecular risk assessment, and the development of representative diagnostic and food safety reference materials.

☆ This article is part of a Special issue entitled: 'Application of Proteomics Techniques' published in International Journal of Biological Macromolecules.

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https://doi.org/10.1016/j.ijbiomac.2026.153941
Received 30 April 2026; Received in revised form 23 July 2026; Accepted 3 August 2026
Available online 8 August 2026
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1. Introduction

Food allergy represents a growing burden worldwide, with prevalences reaching up to 10% of the general population in some regions [1]. This rise is accompanied by geographic variability in sensitisation patterns and dietary exposures, highlighting the need for region-specific identification and characterisation of allergenic food sources [2]. Fish and shellfish (seafood) are among the eight major triggers of food allergy globally according to the Food and Agriculture Organisation of the United Nations (FAO) and World Health Organisation (WHO) [3]. The diagnosis and management of seafood allergies are highly complex and challenging with most affected individuals suffering for a lifetime from strict dietary restrictions and high risks of life-threating anaphylaxis [4–6]. Fish, in particular, is one of the most widely consumed seafood commodities globally. Aquaculture production, dominated by fish in the Asia-Pacific region has rapidly expanded, accounting for 92% of global output and largely driven by Eastern and Southeastern Asia [7,8].

Omics technologies, particularly proteomics, have emerged over recent decades as a powerful analytical platform for comprehensive allergen detection and quantification [9–12]. Proteomics offers the potential to overcome key challenges associated with traditional food allergen testing methods, such as enzyme-linked immunosorbent assay (ELISA). While these methods remain widely utilised for routine monitoring, they are limited by antibody specificity, epitope accessibility, and difficulty in detecting isoforms or unexpected allergens [13,14]. In contrast, a proteomics approach provides a comprehensive overview of the extracted proteome, allowing for the tracing of molecular-level changes. When applied to allergens such as fish, this is particularly advantageous as both the specific identity of allergenic proteins and their quantity affect the overall allergenicity. Furthermore, while ELISA is a strictly targeted approach, proteomics enables deep characterisation of changes induced by food processing, such as protein loss due to aggregation or the enrichment of certain proteins in the extract. Mass spectrometry (MS)-based proteomics such as liquid chromatography (LC)-MS/MS enables unbiased, high-resolution quantification of allergenic proteins (allergens), identification of isoform differences, and allows robust detection of allergens in complex food matrices and processed samples [15,16]. These capabilities position proteomics as an essential tool not only for allergen discovery, but also for molecular risk assessment and quality control, thereby aligning with the objectives of standardised regulatory frameworks for allergen characterisation.

The detection and characterisation of allergens in seafood is complicated by its extensive species diversity and varied expression and isoform diversity of its numerous allergenic proteins [17]. Unlike many common food allergens, fish allergenomes (defined here as the comprehensive repertoire of recognised WHO/International Union of Immunological Societies (IUIS)-registered or candidate fish allergens profiled based on identification and quantification within each species) are shaped by multiple factors, including strong species specificity and proteome-level variation [18,19]. Currently, 12 fish allergens (parvalbumin, β-enolase, aldolase A, tropomyosin, vitellogenin, collagen, creatine kinase, triosephosphate isomerase, pyruvate kinase PKM-like, L-lactate dehydrogenase, glucose 6-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase) across 21 fish species are recognised by the WHO/ IUIS Allergen Nomenclature Sub-committee [20]. Although parvalbumin, a calcium-binding protein, is widely recognised as the dominant fish pan-allergen driving broad cross-reactivity, numerous studies have identified additional allergens with marked species specificity, diverse isoform profiles, and variable abundance across taxa [21–25]. Nevertheless, the majority of existing fish allergen studies have focused predominantly on European and North American species, which represent only a small fraction of the over 2000 fish species consumed globally [26]. This geographic and taxonomic bias limits the generalisation of current allergen datasets and the understanding of region-specific allergen profiles in underrepresented regions such as Asia-Pacific, where consumption and sensitisation patterns differ markedly [27]. Moreover, previous investigations have reported considerable variability in allergen profiles and content across commercial diagnostic extracts used for skin prick testing (SPT), reflecting differences in species selection and extract preparation strategies [28].

In this study, eight fish species were selected to represent a regionally relevant panel of commercially important and widely consumed fish across the Asia-Pacific region, including major aquaculture species (Asian seabass, orange-spotted grouper, Malabar red snapper, and golden pomfret), commonly consumed coastal species (black and white threadfin), globally traded freshwater sutchi catfish, which is extensively farmed and consumed throughout Vietnam and Bangladesh, and nutritionally valuable Atlantic saba mackerel, widely consumed across East Asia (Fig. 1) [7,27,29]. Together, these species capture diverse taxonomic groups, market categories, aquaculture relevance, and expected allergenome variability, thereby providing a regionally relevant basis for evaluating whether current reference extracts and diagnostic panels adequately reflect local seafood exposure.

Aside from species diversity, fish allergens exhibit diverse biochemical properties and varying degrees of heat stability [30,31]. This is particularly significant given the diverse forms in which fish is consumed, ranging from raw preparations to various thermal treatments. Thermal processing induces complex molecular changes. While allergens like parvalbumin are heat-stable, other proteins undergo denaturation that can either destroy conformational epitopes or expose new epitopes. Furthermore, heat-induced protein aggregation can lead to increased resistance to gastrointestinal digestion, potentially enhancing allergenicity by allowing intact allergens to reach the immune system [32]. Conversely, in some instances, cooking may increase digestibility, illustrating that different allergenic proteins behave disparately under thermal stress. Given the widespread consumption of cooked fish and processed fish products, it is important to understand how thermal processing modulates allergen abundance and composition to ensure accurate risk assessment of fish allergens and inform the development of representative diagnostics allergen extract preparations.

Collectively, these factors highlight significant gaps in the molecular characterisation of fish allergens that compromise the accurate representation of regionally relevant allergenomes. As global fish consumption continues to rise, improving the molecular understanding of fish allergen composition has become increasingly important for food safety, clinical diagnosis, and consumer guidance. Despite the widespread application of proteomics in food allergy investigations, comprehensive quantitative proteomics studies examining allergen variability across fish species remain limited [27,33–35]. Unlike previous studies that have primarily focused on a few reference species, individual allergens, or single processing conditions, this is the first study to our knowledge to systematically compare the relative abundance of fish allergens across multiple species using LC-MS/MS proteomics with parallel evaluation of two distinct thermal processing methods. An integrated workflow combining immunological, proteomic, and bioinformatic approaches was developed and applied to characterise fish allergenomes. This design enables simultaneous assessment of species-specific allergenomes, processing-dependent changes in protein extractability, and the identification of potential allergens. By generating allergen-resolved molecular profiles across species and processing conditions, this study provides a translational framework for improving fish allergen risk assessment, supporting the development of more standardised diagnostic approaches, and enabling evidence-based, region-specific clinical management strategies to promote dietary guidance for fish-allergic individuals.

2. Methods

2.1. Sampling

Three fresh whole specimens each of eight fish species: sutchi catfish (swai; *Pangasianodon hypophthalmus*), Asian seabass (barramundi; *Lates*

calcarifer), white threadfin (*Eleutheronema tetradactylum*), black threadfin (*Leptomelanosoma indicum*), golden pomfret (*Trachinotus blochii*), Malabar red snapper (*Lutjanus malabaricus*), orange-spotted grouper (*Epinephelus coioides*), and Atlantic saba mackerel (*Scomber scombrus*) were sourced from a local seafood supplier in Singapore, hereafter referred to as catfish, seabass, white threadfin, black threadfin, pomfret, snapper, grouper, and mackerel, respectively. Species identification was confirmed based on morphological features, supported by polymerase chain reaction and Sanger sequencing [36]. Standardised muscle samples were excised from predefined anatomical locations in each specimen, as detailed previously [37], and samples from the three specimens were pooled to reduce biological variation, generating one experimental unit per species [38]. The overall experimental and analytical workflow, including downstream sample preparation, immunological analyses, LC-MS/MS proteomics, WHO/IUIS allergen annotation and *in silico* allergenicity prediction, is summarised in Fig. 2.

2.2. Protein extraction and quantification

For the preparation of raw extracts (RE), minced raw muscle tissues were homogenised in chilled phosphate-buffered saline (PBS) with a rotor-stator homogeniser (20 s at 60,000 rpm on ice) using a sample-to-buffer ratio of 1:3 (*w/v*) as established previously [30]. Proteins were extracted overnight with gentle agitation at 4 °C, followed by centrifugation at 20,000 x*g* for 20 min. Heat treatment was evaluated in two ways: Heated extracts (HE) were prepared by heating clarified raw extracts at 95 °C for 10 min, followed by centrifugation at 20,000 x*g* for 20 min. Heated tissue (HT) extracts were prepared by heating raw tissue in PBS at 90–95 °C for 20 min. Similar to the RE preparation, heated muscle tissues were homogenised in PBS, extracted overnight with gentle agitation at 4 °C, followed by centrifugation at 20,000 x*g* for 20 min. All extract preparations were subjected to a standardised freeze-thaw cycle, followed by centrifugation to remove any precipitation or insoluble material. Proteins solubilised in clarified supernatant of the three preparations were quantified using the Pierce™ Dilution-Free™ Rapid Gold BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin standards according to the manufacturer's instructions for downstream analysis.

2.3. Total protein profiling and immunoblot analysis

Proteins were resolved by Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using Criterion™ TGX Stain-Free™ AnykD precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) following manufacturer's instructions. Protein bands were visualised by Coomassie Brilliant Blue R-250 staining and gels scanned with an Odyssey M® imaging system (LI-COR Biosciences, Lincoln, NE, USA). Bands containing fish allergens were estimated based on their molecular weight and mass spectrometric analyses after in-gel tryptic digestion in previous studies [13,22,25]. Identification of allergenic protein bands and other IgE-binding proteins was performed using immunoblot as previously described [39]. In brief, nitrocellulose membranes containing transferred proteins were incubated with monoclonal anti-parvalbumin antibody PARV-19 (Sigma-Aldrich, St. Louis, MO, USA) or pooled patient sera ($n = 6$) from clinically confirmed IgE-mediated allergy paediatric subjects [22], followed by monoclonal anti-human IgE antibody (sc-53,346 by Santa Cruz Biotechnology, Dallas, TX, USA). Ethical approval was obtained from the Sydney Children's Hospitals Network (LNR-14/SCHN/185) and written informed consent was provided. Immunofluorescence secondary antibody DyLight 800 goat anti-mouse (H + L) 4× PEG (Invitrogen, Carlsbad, CA, USA) was used to develop the blots followed by imaging using the aforementioned Odyssey M system. Densitometric analysis of bands at 10–15 kDa, corresponding to the expected molecular weight range of parvalbumin, was performed using the Image Studio v6.0 software. Signal intensities were summed for species with more than one band within the region.

2.4. Mass spectrometry data acquisition

Protein extracts were subjected to tryptic digestion using S-Trap™ micro spin column (ProtiFi, Farmingdale, NY, USA) following manufacturer's instructions as described previously [40]. Technical reproducibility of the LC-MS/MS workflow was assessed by analysing the raw catfish extract in triplicate under identical acquisition as all samples. The resulting triplicate measurements represent technical replicates and were not treated as independent biological observations. Tryptic peptides were analysed on a Dionex UltiMate 3000 RSLC system (Thermo Scientific-Dionex Softron GmbH, Germering, Germany) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nano-electrospray ionisation source

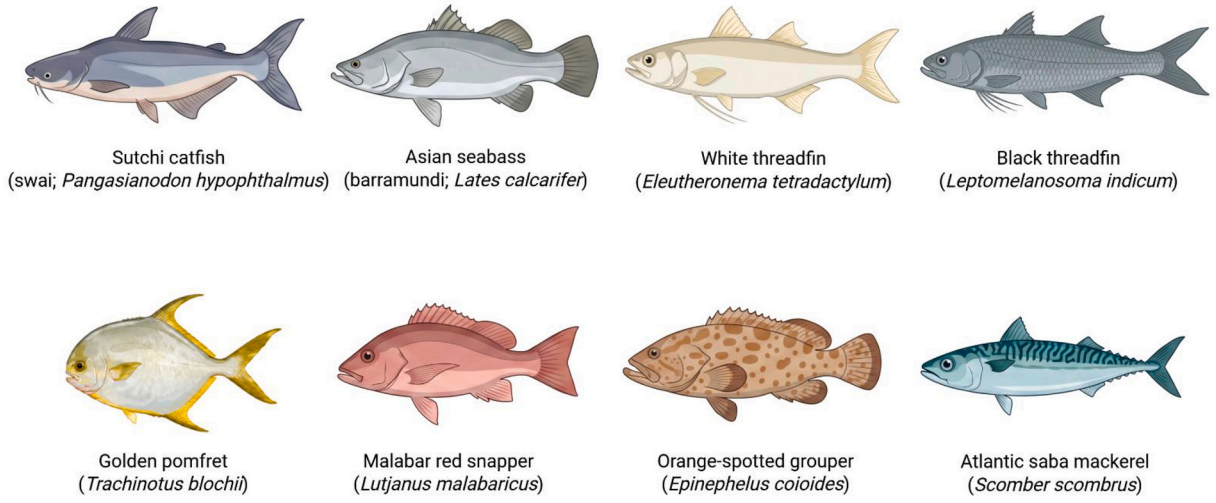


Fig. 1. Fish species selected for the allergen profiling study.
Eight commercially and regionally relevant fish species were selected to represent commonly consumed species across the Asia-Pacific region [7,27,29]. The species analysed were sutchi catfish (swai; *Pangasianodon hypophthalmus*), Asian seabass (barramundi; *Lates calcarifer*), white threadfin (*Eleutheronema tetradactylum*), black threadfin (*Leptomelanosoma indicum*), golden pomfret (*Trachinotus blochii*), Malabar red snapper (*Lutjanus malabaricus*), orange-spotted grouper (*Epinephelus coioides*), and Atlantic saba mackerel (*Scomber scombrus*).

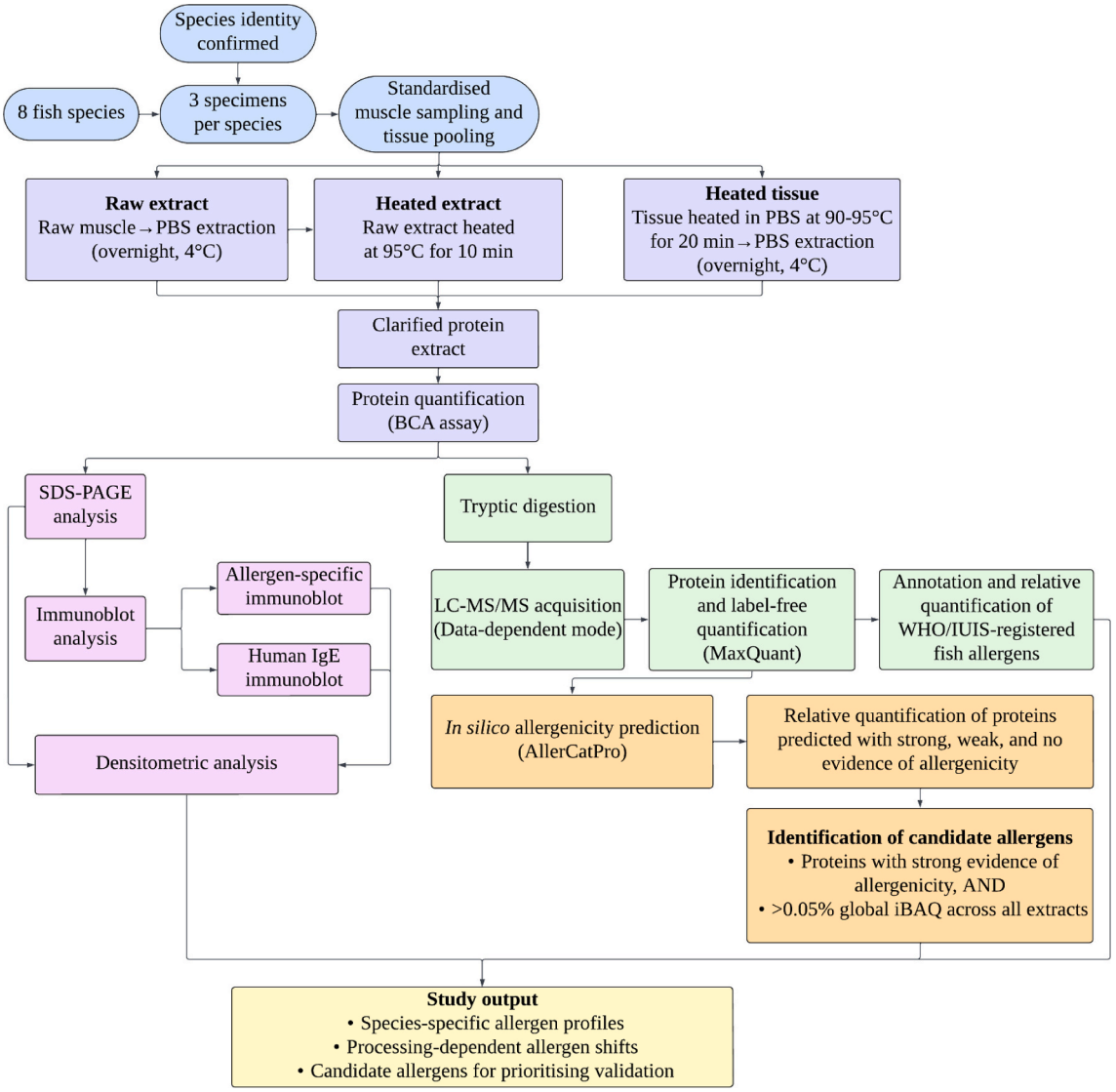


Fig. 2. Experimental and analytical workflow for integrated fish allergen profiling.
Eight fish species were analysed after confirming species identity and sampling standardised muscle cuts from three specimens per species. Pooled muscle tissues were subjected to three preparation states: raw extract, heated extract and heated tissue. Clarified protein extracts were quantified and analysed by SDS-PAGE, immunoblotting, densitometry and LC-MS/MS-based proteomics. Protein identification and label-free quantification were performed using MaxQuant and *in silico* allergenicity prediction using AllerCatPro. Candidate allergens were prioritised based on strong AllerCatPro evidence and > 0.05% global iBAQ across all preparation states. The integrated workflow enabled assessment of species-specific allergen profiles, processing-dependent allergen redistribution and candidate allergens for future validation.

and operated in positive ion mode. The ion transfer tube temperature was set to 275 °C and spray voltage was 1.9 kV. Peptides were first concentrated on an Acclaim PepMap nano trap column (Dionex-C18, 100 Å, 75 μm × 2 cm) and then separated on an Acclaim PepMap RSLC analytical column (Dionex-C18, 100 Å, 75 μm × 50 cm). Peptides were eluted at a flow rate of 300 nL/min using a multistep gradient of solvent B: 3% at 6 min, 23% at 65 min, 40% at 75 min, and 80% at 80 min, held until 85 min, followed by re-equilibration at 3% B to 95 min. Full MS spectra were acquired over the range *m/z* 300–1600 at a resolution of 120,000 with a maximum injection time of 25 ms and a normalised AGC target of 300%. Peptide ion fragmentation was carried out using data-dependent acquisition performed in cycle-time mode (3 s) using monoisotopic precursor selection. Precursor ions were filtered with a minimum intensity threshold of 5000 and charge states of 2–6 and were subjected to dynamic exclusion for 20 s with a ± 10 ppm mass tolerance. MS/MS spectra were acquired in the Orbitrap using an isolation window of *m/z* 1.2, higher energy collisional dissociation at 30% normalised collision energy, a resolution of 15,000, a first mass of *m/z* 120, and a normalised AGC target of 75%.

2.5. Mass spectrometric analysis

Protein identification and label-free quantification (intensity-based absolute quantification [iBAQ]) were performed using MaxQuant search engine v2.6.7 against carefully selected species-specific databases as detailed in Table 1 [41,42]. MMTS (C) was set as a fixed modification, oxidation (M) and acetyl (protein N-term) as variable modifications, and trypsin/P as the proteolytic enzyme with maximum of two missed cleavages allowed. Protein identification was controlled at a 1% false discovery rate on peptide-spectrum match (PSM) and protein levels. Based on fish allergens recognised by WHO/IUIS, 11 fish allergen groups, namely parvalbumin, enolase, aldolase, tropomyosin, collagen, creatine kinase, triosephosphate isomerase (TPI), pyruvate kinase, L-lactate dehydrogenase(DH), glucose-6-phosphate isomerase (GPI), and

glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were established based on protein names. The relative percentage abundance of each protein within a sample was calculated by dividing the iBAQ value of each protein by the summed iBAQ value, hereafter referred to as iBAQ% [22]. MaxQuant-derived iBAQ% for raw catfish replicates were summarised as mean and standard deviation and are provided in Table B.1.

2.6. In silico allergenicity prediction

All proteins identified by MS were subjected to *in silico* allergenicity prediction using AllerCatPro 2.0 [43]. Prediction was used to complement the WHO/IUIS allergen nomenclature database by identifying not only recognised fish allergens, but also proteins not yet registered that showed structural similarity or epitope-related similarity to known allergens reported in the FARRP, COMPARE, UniProtKB, and Allergome databases. The relative abundance (iBAQ%) of query proteins were summed based on their classification as strong, weak, or no evidence of allergenic potential in AllerCatPro. For downstream candidate prioritisation, analyses were restricted to proteins classified as having strong evidence of allergenicity and contributing >0.05% global iBAQ across all preparation states, which are considered as positive predictions. This threshold was selected following sensitivity analyses comparing >0.1%, >0.05% and > 0.01% global iBAQ cut-offs, with >0.05% providing the most balanced retention of processing-associated candidate allergens while limiting the inclusion of trace-level proteins.

Positively predicted proteins were grouped by their common allergen or protein family names based on WHO/IUIS-registered fish allergens and non-WHO/IUIS-registered candidate allergenic proteins, hereafter referred to as fish allergens and candidate allergens (or candidates), respectively, combining corresponding isoforms, variants, and subunits where appropriate. The relative abundance of each allergen group was then calculated by summing the iBAQ% values of all corresponding proteins within the protein group.

2.7. Statistical analyses

For visualization of species-specific protein and allergen patterns, iBAQ% values were *Z*-score normalised and subjected to unsupervised hierarchical clustering using Perseus software v2.1.5 [44].

Processing-induced changes were evaluated using $\log_2$-transformed relative abundance ratios were performed using GraphPad Prism v8.4.3, for heated extract *versus* raw extract (HE/RE), heated tissue *versus* raw extract (HT/RE), and heated extract *versus* heated tissue (HE/HT), calculated as follows:

$$\log_2\left(\frac{HE\ or\ HT}{RE}\right) = \log_2\frac{iBAQ\%(HE\ or\ HT)}{iBAQ\%(RE)}$$

$$\log_2\left(\frac{HE}{HT}\right) = \log_2\frac{iBAQ\%HE}{iBAQ\%HT}$$

Values close to 0 indicate little change between the compared conditions, positive values indicate relative enrichment in the numerator condition, and negative values indicate relative enrichment in the denominator condition. Accordingly, positive $\log_2$ (relative abundance ratio, HE/HT) values indicate greater relative abundance in HE, whereas negative values indicate greater relative abundance in HT.

3. Results and discussion

3.1. Comparative total protein and parvalbumin profiles and IgE-binding patterns

SDS-PAGE analysis demonstrated distinct species-specific and extractable total protein profiles, and allergenomes following thermal processing (Fig. 3). In RE, all species exhibited complex banding patterns, with prominent bands observed in the ~100 kDa (collagen), ~60 kDa (GPI and pyruvate kinase), 35–50 kDa (L-lactate DH, GAPDH, tropomyosin, aldolase, creatine kinase, and enolase), ~25 kDa (TPI), and 10–15 kDa (parvalbumin) regions. Clear interspecies variability was evident in both band intensity and distribution, reflecting intrinsic biological differences in muscle proteomes across taxa. Most species showed prominent parvalbumin bands, while mackerel exhibited comparatively weaker intensity, suggesting differences in major allergen abundance and lower allergenicity as indicated previously [45,46]. Densitometric quantification and immunoblot analyses (Table 2) confirmed interspecies variability in parvalbumin-associated signal intensity in RE, consistent with SDS-PAGE observations, with the highest signals observed in grouper (Band intensity: 2570; Parvalbumin detection: 40,500) and the lowest in mackerel (46; 585). Notably, variation in the number of parvalbumin bands suggests species-specific and isoform differences, with catfish, seabass, white threadfin and snapper exhibiting two distinct bands, whereas the other species displayed a single band within the ~10–15 kDa region [21]. IgE immunoblots showed additional bands detected between ~37–60 kDa across several species, likely representing creatine kinase, aldolase, tropomyosin, enolase, pyruvate kinase, or GPI. A distinct ~22 kDa band was recognised in both parvalbumin-specific and IgE-binding immunoblots of white threadfin, suggesting the presence of a heat-stable, immunoreactive protein (Fig. A.1 and A.2). This band may represent a parvalbumin oligomer (*e.g.* dimer) or a species-specific protein contributing to its distinct extractable proteome [47].

Closely related species such as black and white threadfin (family Polynemidae) showed visibly distinct total protein profiles, indicating that taxonomic proximity alone is insufficient to predict allergen composition and clinical representation. Allergen composition appears to be strongly species-dependent, even among related taxa. Importantly, white threadfin exhibited disproportionately elevated parvalbumin-specific and IgE-binding signal intensities despite lower overall band intensity compared to black threadfin, consistent with its higher relative parvalbumin abundance (Table 2). This suggests that total protein abundance does not always correlate with allergen-specific concentration. A specific allergen may represent a greater relative proportion of the total proteome in one species or possess higher immunoreactivity even at lower absolute levels. Together, these findings highlight the

Table 1
Species-specific protein databases used for proteomic identification across eight fish species. Protein identification was conducted using species-specific or taxonomically resolved protein sequence databases selected based on availability and annotation completeness.

Common name	Scientific name	Database	Source	Number of entries	Resolution
Sutchi catfish	*Pangasianodon hypophthalmus*	Reference Sequence	NCBI	47,394	Species
Asian seabass	*Lates calcarifer*	UniProtKB	UniProt	57,547	Species
White threadfin	*Eleutheronema tetradactylum*	Reference Sequence (Carangaria *incertae sedis*)	NCBI	84,023	Higher taxon
Black threadfin	*Leptomelanosoma indicum*	Reference Sequence (Carangaria *incertae sedis*)	NCBI	84,023	Higher taxon
Golden pomfret	*Trachinotus blochii*	UniProtKB (Carangidae)	UniProt	69,150	Higher taxon
Malabar red snapper	*Lutjanus malabaricus*	In-house proteome	[42]	23,894	Species
Orange-spotted grouper	*Epinephelus coioides*	Protein database	NCBI	41,743	Species
Atlantic saba mackerel	*Scomber scombrus*	UniProtKB	UniProt	35,480	Species

Note: Where species-specific databases were unavailable or incomplete, higher-level taxonomic databases were used to maximise protein identification coverage.

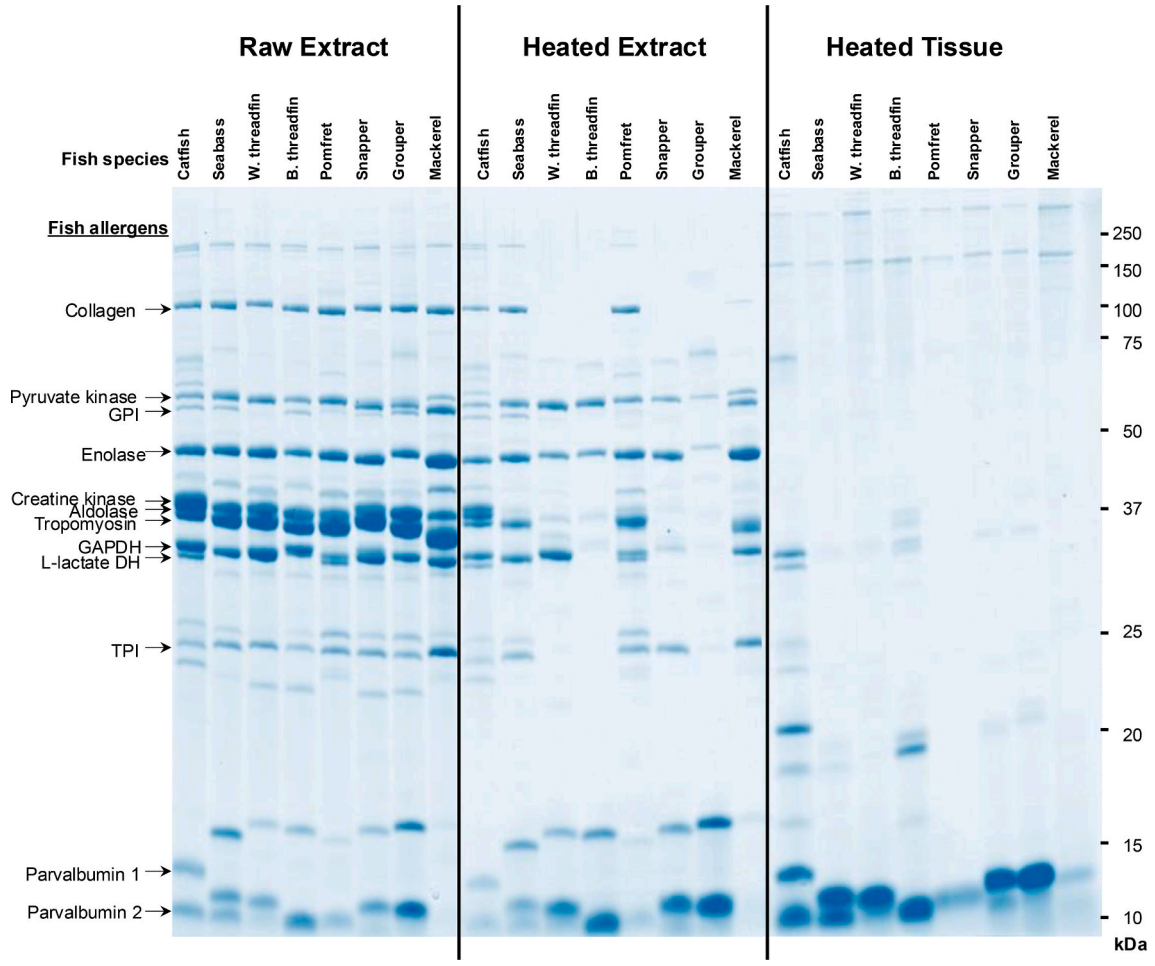


Fig. 3. Comparative SDS-PAGE profiles across eight fish species under three preparation conditions.
Total protein profiles of raw extract (RE), heated extract (HE), and heated tissue (HT) are shown for sutchi catfish, Asian seabass, white threadfin, black threadfin, golden pomfret, Malabar red snapper, orange-spotted grouper, and Atlantic saba mackerel. Note: The bands primarily containing the 11 fish muscle allergens are indicated with an arrow as established previously [22,37]. GPI, glucose-6-phosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DH, dehydrogenase; TPI, triosephosphate isomerase.

Table 2
Densitometric analysis of parvalbumin bands, their recognition by allergen-specific and human IgE antibodies, as well as their relative abundance (iBAQ%). Total signal within the ~10–15 kDa parvalbumin-associated molecular weight region in the SDS-PAGE profile (Fig. 3), parvalbumin-specific and patient IgE-binding immunoblot assays (Fig. A.1 and A.2, respectively), as well as proteomics-based abundance. Percentage values indicate the relative contribution or proportion of detected signal under the corresponding condition. Note: n.d., not detected.

		Band intensities (SDS-PAGE)			Signal intensities						Relative abundance (iBAQ%)		
					Parvalbumin detection			IgE-binding					
	Condition	Raw Extract	Heated Extract	Heated Tissue	Raw Extract	Heated Extract	Heated Tissue	Raw Extract	Heated Extract	Heated Tissue	Raw Extract	Heated Extract	Heated Tissue
Species	Catfish	1,797	525	6,320	15,120	15,400	55,800	135	138	532	25%	11%	55%
	Seabass	2,014	1,087	5,490	20,700	18,440	33,800	64	123	254	36%	11%	71%
	White threadfin	741	1,670	3,140	38,390	42,300	52,900	92	406	242	18%	48%	51%
	Black threadfin	1,570	3,280	3,690	29,000	19,700	16,800	47	122	123	18%	5%	14%
	Pomfret	605	362	2,350	15,900	4,520	16,600	22	n.d.	47	26%	10%	40%
	Snapper	1,129	2,630	4,745	30,500	38,300	17,320	87	119	290	32%	38%	74%
	Grouper	2,570	5,430	7,550	40,500	50,300	28,100	202	180	414	34%	38%	59%
	Mackerel	46	35	501	585	730	1,250	n.d	n.d.	40	5%	6%	57%

importance of an integrated analytical approach, as total protein profiles alone may not accurately reflect allergen-specific abundance or immunoreactive potential. These observations are especially relevant in the Asia-Pacific context, where consumption patterns and clinically relevant exposures differ from those in Europe and North America, yet commercial diagnostic preparations are still commonly based on a limited number of non-regional species such as cod or salmon [28]. As a result, common commercial diagnostic extracts may incompletely reflect the allergenic composition of fish species relevant to local patient sensitisation patterns.

Here, we employed two fundamentally different models of thermal processing and allergen exposure: HT and HE. HT reflects heating within intact muscle matrix, which closely mimics real-world consumption conditions, whereas HE represents heating of already solubilised

proteins, as commonly used in laboratory extraction and diagnostic preparations. These conditions differentially affect protein solubility, stability, and retention, particularly for structurally distinct allergens such as parvalbumin and collagen. Understanding these differences is therefore critical to determine how processing influences allergen composition and IgE-binding, and to evaluate whether current extract-based diagnostic approaches accurately reflect clinically relevant exposure [48,49].

Overall, thermal processing substantially altered the extractable proteome. HE preparations displayed generally reduced band intensities and more selective retention of species-specific proteins. The disappearance of bands in collagen-associated high molecular weight region in HE of threadfins, snapper, grouper, and mackerel suggests differential effects of heating on protein solubility and extractability. Conversely, HT showed reduced overall banding complexity but retained or revealed IgE-binding, particularly in high molecular weight bands corresponding to collagen (Fig. A.2). This observation is consistent with the known biochemical properties of fish collagen, which is poorly soluble in neutral aqueous buffers such as PBS. However, retention of IgE-binding capacity reflects the heat-stability of its allergenic epitopes. At a molecular level, while thermal denaturation typically disrupts conformational (structural) epitopes, it may leave linear epitopes intact or expose previously buried "neoepitopes" through protein unfolding. For collagen, heating within the tissue matrix may denature the collagen triple helix and generate gelatin-like or aggregated structures while preserving IgE-reactive epitopes [25]. This allows heat-stable allergens to maintain their immunoreactive potential even after the loss of their higher-order protein structure [32]. Relative enrichment of parvalbumin-associated molecular weight region across most species was consistent with increased parvalbumin signal intensity, and relative abundance shown in Table 2. This enrichment was accompanied by generally elevated IgE-binding responses in HT compared to RE, indicating enhanced immunoreactivity of heat-stable allergens [50–52]. In catfish, seabass, white and black threadfin, snapper, and grouper, additional bands corresponding to identified allergens such as GAPDH and L-lactate DH, and other proteins at ~20 kDa, remained detectable after tissue heating, which indicates partial persistence of selected proteins despite a marked reduction in overall proteomic complexity. IgE-binding patterns are driven primarily by the relative distribution of heat-stable allergens rather than total protein complexity. Our findings are consistent with previous work using a comparable HT extraction approach, which demonstrated IgE-binding to parvalbumin and/or collagen across six fish species with varying signal intensities [53].

Our findings also highlight important limitations associated with the exclusive use of raw extracts in current diagnostic workflows. While raw extracts are commonly used in SPT, they may not adequately represent the allergen profile encountered during consumption, where fish is typically thermally processed. The diverging outcomes of this study suggest that allergen composition and immunoreactivity are strongly influenced by processing context, and that raw extracts alone may alter the visibility of key allergens, ultimately underestimate or misrepresent clinically relevant allergen exposure. Crucially, traditional diagnostic extracts often fail to capture the full proteomic diversity of the fish. While an extract only contains soluble components, the consumer is exposed to the entire fish proteome, including insoluble or matrix-bound proteins that may still possess significant immunoreactivity. Incorporating processing-informed extract preparation or complementary approaches may therefore improve the sensitivity and relevance of diagnostic testing, particularly for populations with high consumption of cooked seafood such as Asia-Pacific. In this context, the application of proteomics provides a powerful advantage, enabling high-resolution characterisation of these complex molecular shifts that traditional assays miss. By mapping the "total" rather than just the "soluble" proteome, proteomics ensures a more impactful and comprehensive assessment of allergen risk. Collectively, these qualitative profiles established that both species identity and processing state strongly influence the composition of fish protein extracts, providing a qualitative foundation for subsequent quantitative allergenomic analyses.

3.2. Distinct species-specific allergen profiles

LC-MS/MS profiling identified 529–1012 protein groups across the eight species, confirming substantial heterogeneity in protein composition. Vitellogenin was not detected in any samples, consistent with its role as a tissue-specific yolk precursor protein and its absence in fish muscle tissue [54–56]. Hierarchical clustering of *Z*-score normalised iBAQ% resolved distinct species groupings based on quantitative allergen distribution in RE (Fig. 4). Parvalbumin was dominant in five of the eight species with widely varying relative abundance. Highest proportions were observed in catfish (25%), seabass (36%), grouper (34%), snapper (32%), and pomfret (26%), whereas mackerel showed the lowest parvalbumin abundance (5%) (Table 2). In both threadfin species, creatine kinase exceeds parvalbumin abundance.

Two major allergen clusters were observed. The first comprised of black and white threadfin together with catfish, characterised by co-dominance of parvalbumin and creatine kinase, accompanied by decreased representation of muscle-associated proteins such as tropomyosin and collagen, and certain metabolic enzymes. The second cluster included seabass, grouper, and snapper, which also showed consistently high relative abundance of parvalbumin and creatine kinase, with pomfret showing close similarity within this group. In contrast, mackerel formed a distinct outlier cluster defined by low parvalbumin abundance and a greater contribution of metabolic enzymes, including enolase, aldolase, and TPI. This pattern indicates a fundamentally different allergen distribution from the parvalbumin-rich species.

Across all species, the allergenomes could be broadly segregated into three functional groups: (i) dominant allergens, represented primarily by parvalbumin and creatine kinase, (ii) metabolic enzymes, including enolase, aldolase, TPI, L-lactate DH, GAPDH, and (iii) low-abundance structural proteins such as tropomyosin and collagen, as well as metabolic enzymes including GPI and pyruvate kinase. This stratification

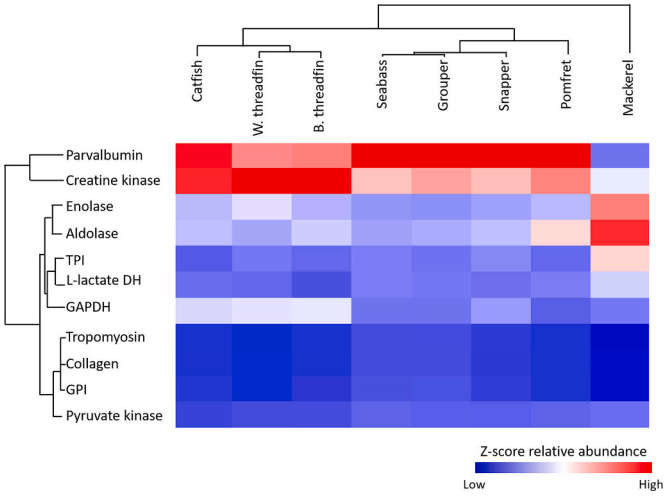


Fig. 4. Hierarchical clustering heatmap of quantitative allergen profiles in raw extracts across eight fish species.
Unsupervised hierarchical clustering of *Z*-score-normalised relative allergen abundance (iBAQ%) across sutchi catfish, Asian seabass, white threadfin, black threadfin, golden pomfret, Malabar red snapper, orange-spotted grouper, and Atlantic mackerel. Red and blue indicate relatively higher and lower abundance, respectively, after row-wise normalisation. The heatmap highlights species-specific variation in the proportional distribution of recognised fish allergen groups. TPI, triosephosphate isomerase; DH, dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPI, glucose-6-phosphate isomerase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

highlights conserved functional classes of fish allergens alongside marked species-specific variation in their proportional abundance.

These findings reinforce the central role of parvalbumin as the major fish pan-allergen and likely principal driver of broad IgE cross-reactivity [35,57]. However, they also show that parvalbumin dominance is not uniform across species. Relative abundance varied by up to 7-fold in RE, with mackerel displaying a uniquely low-abundance profile (Table 2), suggesting that allergenicity is influenced not only by the presence of a known pan-allergen, but also by its quantitative dominance relative to other allergenic proteins. In species where parvalbumin contributes a smaller proportion of the extractable proteome, additional allergens or isoforms may play a more prominent role in shaping sensitisation patterns [24]. This is relevant not only for clinical diagnosis but also for analytical detection strategies. Current fish allergy diagnostics frequently rely on a limited number of highly studied species, particularly Atlantic cod (*Gadus morhua*), in which the major β-parvalbumin (Gad m 1) is abundant and highly immunogenic [58]. Consequently, these commercial extracts may not adequately represent distinct molecular allergenomes, as observed here in species such as mackerel, which can contribute to false-negative or weak SPT outcomes in clinically reactive individuals [59]. This problem is especially important in the Asia-Pacific region, where dietary exposure is characterised by a diverse range of locally consumed fish species that are poorly represented in current diagnostic panels. Similarly, ELISA-based allergen detection methods rely on antibody recognition of a single target allergen or epitope (most often parvalbumin), are inherently biased during assay development. Such approaches may fail to capture the full allergenic complexity of low-parvalbumin species or those in which other proteins contribute substantially to allergenicity [13]. Together, these results support the need for a more quantitative and species-resolved framework for fish allergen assessment rather than a single presence-or-absence approach centred solely on parvalbumin.

3.3. *Heat processing effects on soluble protein abundance and allergen extractability*

Abundance changes in HE and HT relative to RE, as well as direct HE/HT comparisons were used to quantify processing impacts (Fig. 5). Notably, heating did not produce a single uniform processed fish allergenome. Instead, the two heating approaches generated distinct post-processing profiles. Parvalbumin remained one of the most thermostable allergens across the panel, with broadly retained or increased abundance after heating.

In Fig. 5A, HE preparations showed a relatively variable and species-dependent pattern across most species. Although some proteins showed the expected loss after heating, several apparently heat-labile proteins,

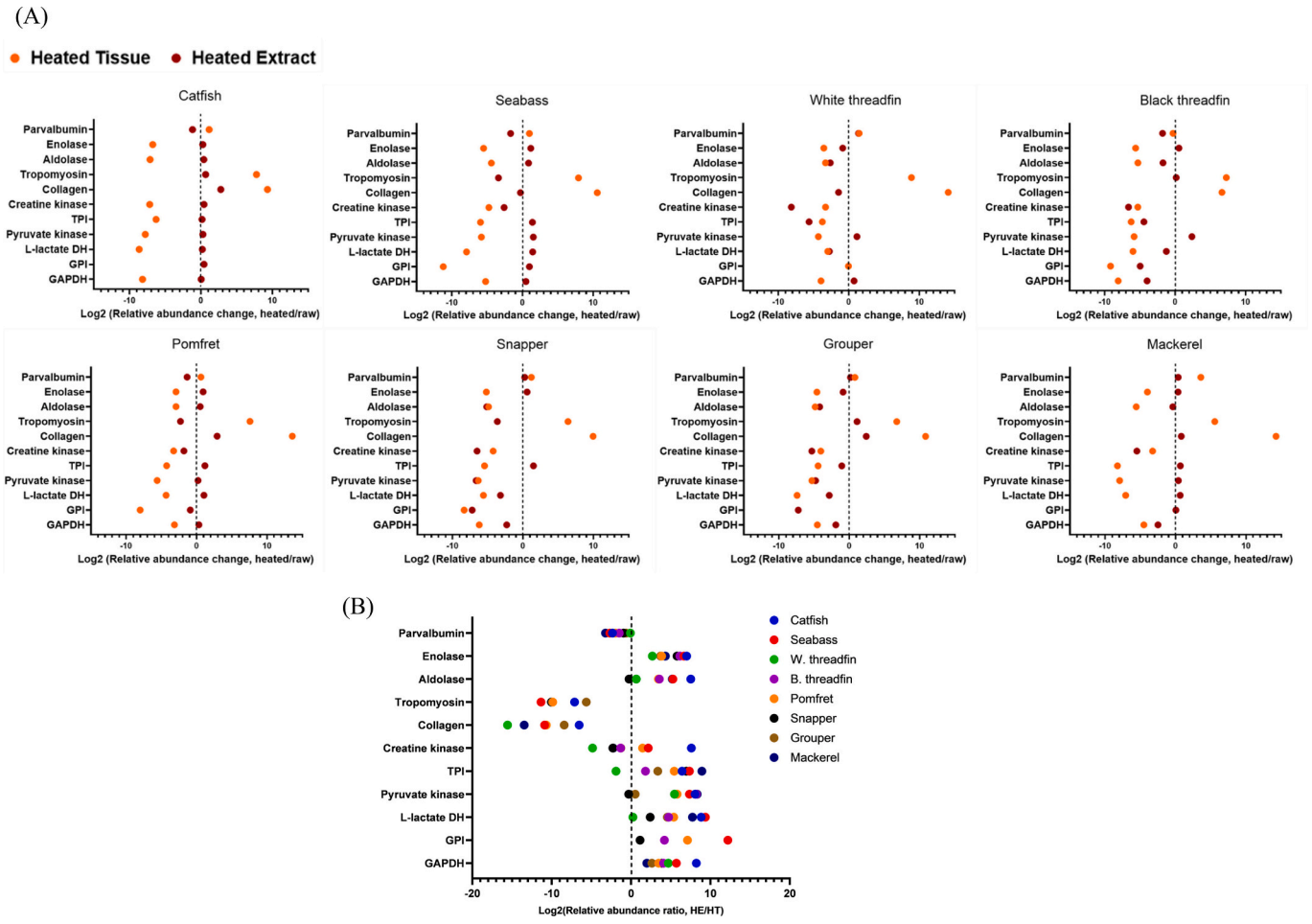


Fig. 5. Processing-induced changes in relative allergen abundances due to differential heat treatments.
(A) Log2-transformed relative abundance changes of fish allergen groups in heated extract (HE) and heated tissue (HT) compared with raw extract (RE) for each species. Positive and negative values indicate relative enrichment and depletion compared with RE, respectively. (B) Direct comparison of heated preparation workflows expressed as log2 relative abundance ratio (HE/HT) across all species. Positive values indicate relative enrichment in HE, whereas negative values indicate relative enrichment in HT. Note: Missing data points indicate that abundance ratios could not be calculated because the protein was not quantified in one or both paired preparation states. TPI, triosephosphate isomerase; DH, dehydrogenase; GPI, glucose-6-phosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

particularly metabolic enzymes, were retained or even relatively enriched in the soluble HE fractions. In catfish, seabass, pomfret, and mackerel, metabolic enzymes such as enolase, aldolase, TPI, L-lactate DH, and GAPDH showed a consistent increase in HE while substantially reduced or lost in HT. In contrast, tropomyosin and collagen showed only modest retention in HE but strong enrichment in HT. These contrasting outcomes indicate that the effects of heating on allergen composition are governed not only by intrinsic thermal stability but by complex matrix interactions.

Mechanistically, heating disrupts the non-covalent interactions that stabilise protein structure, leading to partial unfolding and exposure of hydrophobic regions. This can promote intermolecular protein association through hydrophobic interactions, disulfide exchange, and subsequent aggregation. In muscle systems, many sarcoplasmic proteins, including metabolic enzymes such as enolase, aldolase, and creatine kinase, are susceptible to heat-induce aggregation, while structural proteins such as tropomyosin and collagen undergo unfolding and protein-protein association, contributing to structural reorganisation of the tissue matrix [60]. Under the HT condition, these changes occur within a dense cellular environment in which proteins remain embedded in the intact myofibrillar and connective tissue architecture. The high local protein density, connective tissue restructuring, water loss and matrix contraction can facilitate protein-matrix trapping, where denatured proteins aggregate and co-precipitate with the insoluble cooked tissue structure, leading to their apparent loss from the extractable fraction. This model closely mimics domestic preparation of cooked fish muscle for consumption and captures the influence of matrix effects on allergen exposure.

In contrast, heating a pre-prepared extract (HE) represents heating of proteins that have already been solubilised and separated from intact tissue structure, and therefore more closely resembles laboratory or diagnostic extract preparation. The preparation of HE involves a much lower protein density. Without the surrounding tissue matrix, these same proteins may undergo structural unfolding or partial denaturation without becoming entrapped within an insoluble structural network. Consequently, proteins that precipitate during heating are removed during clarification whereas proteins that remain soluble retained in the HE despite minor aggregation. This provides a plausible explanation for why selected metabolic proteins were retained or relatively enriched in HE but substantially depleted in HT. Nevertheless, strongly heat-stable allergens such as parvalbumin confer great resistance to irreversible denaturation, precipitation and matrix entrapment attributed by its small and compact calcium-binding structure compared to larger sarcoplasmic proteins or metabolic enzymes [61,62]. Therefore, the resulting allergenic profile is a product of both the protein's thermal response and its physical partitioning between the soluble extract and the insoluble tissue matrix. As such, extracts prepared from soluble RE or HE fractions may not reflect the allergen profiles encountered in cooked fish tissue, particularly tropomyosin, collagen, and other allergens retained within matrix. In addition, common analytical risk assessment methods including ELISA-based approaches developed around parvalbumin or other extract-soluble proteins may under-represent matrix-retained allergens. Reference materials and proteomic markers accounting for processing effects may therefore improve the representativeness of diagnostic and food safety assessments for fish products [16].

Direct comparison of HE/HT ratios further resolved two broad physicochemical classes of proteins (Fig. 5B). Tissue-retained proteins, represented most clearly by parvalbumin and structural proteins such as tropomyosin and collagen, preferentially accumulated and retained in HT and were underrepresented in soluble HE. Conversely, extract-soluble proteins, including enolase, aldolase pyruvate kinase, L-lactate DH, GPI, and GAPDH, were more strongly represented in the soluble post-heating fraction. Some proteins, including creatine kinase and TPI showed more balanced behaviour depending on species. Together, these results indicate that the cooked fish allergenome is shaped by both thermal stability and matrix-dependent extractability, and that these are related but not interchangeable properties [63].

More importantly, protein enrichment after tissue heating should be interpreted primarily as relative enrichment resulting from the depletion of more labile proteins, hence only as relative accumulation of some allergens. These observations reveal that the processed allergenome is not a fixed entity. Hence, concluding that a fish allergen is simply "heat-stable" or "heat-labile" is insufficient for accurate risk assessment. Instead, our findings demonstrate that the diagnostic and assessment relevance of an allergen depends on whether it is encountered in a soluble extract or within a complex tissue matrix. This distinction is critical and suggests that relying on a single extraction method may systematically overlook significant allergens that are only "unmasked" or enriched under specific processing conditions.

3.4. Identification of candidate allergens by in silico prediction

The majority of relative abundance remained attributable to the established fish allergenomes, with parvalbumin consistently representing the dominant component of the predicted allergen-associated profiles across all preparation states. Additionally, proteins with either strong or weak evidence of allergenicity were identified and classified by AllerCatPro 2.0, and their relative abundances were summed. Fig. 6 summarises these classified protein profiles, and all proteins with strong AllerCatPro evidence are summarised in Table C.1. for all the analysed species.

In RE, the predicted allergen landscape was still largely driven by parvalbumin, but species differed in the extent to which candidate allergens (other proteins with strong allergenicity evidence) contributed to the remaining allergen fraction. Parvalbumin occupied the largest proportion in all species except black threadfin, white threadfin, and mackerel. The relative contribution of weak evidence allergens was substantially greater in seabass, black threadfin, and grouper. Creatine kinase remained the major secondary contributor in most species, especially the parvalbumin-rich cluster previously identified in the raw allergenome. By contrast, mackerel displayed a less parvalbumin-centred composition and a broader distribution of other protein classes, consistent with its outlier status in the hierarchical clustering of raw allergen profiles. These data align with the overall finding that raw fish allergenomes are strongly species-specific rather than uniformly pan-allergen dominated.

In HE, the relative proportion of fish allergens was reduced in several species and was accompanied by a prominent increase in weak evidence fractions. Black threadfin, in particular, showed drastically increased weak evidence fraction. In HT, parvalbumin dominated most species, but the relative contribution of strong and weak evidence candidate allergens became more apparent in selected species, especially black threadfin and mackerel. Thus, the prediction-based analysis indicates that processing can increase the relative prominence of certain low-abundance candidate allergens even when the overall cooked allergenome remains quantitatively dominated by parvalbumin.

After restricting the analysis to proteins contributing to >0.05% of the global iBAQ across all preparation states, 14 candidate allergens with strong AllerCatPro evidence were prioritised (Fig. 7). Using RE as a baseline (Fig. 7A), phosphoglycerate kinase was the most consistently represented candidate at an appreciable abundance, being detected in all eight species, ranging from 0.5% in grouper to 2.7% in mackerel. Myosin light chain was also recurrent in all eight species, although at a lower relative abundance ranging from 0.1% in seabass, pomfret, white threadfin, snapper, and mackerel to 0.5% in catfish. In contrast, other candidates were much more restricted to specific species, occurring in six or fewer of the tested species at low abundances. Aspartate aminotransferase and malate dehydrogenase reached high relative abundances of approximately 0.7%, respectively. Troponin was restricted to black threadfin (0.1%) and snapper (0.3%), as well as seabass which was detected below MS limit of quantification. Hemoglobin subunit beta was only prominent in catfish (0.8%) and seabass (0.4%). Additional

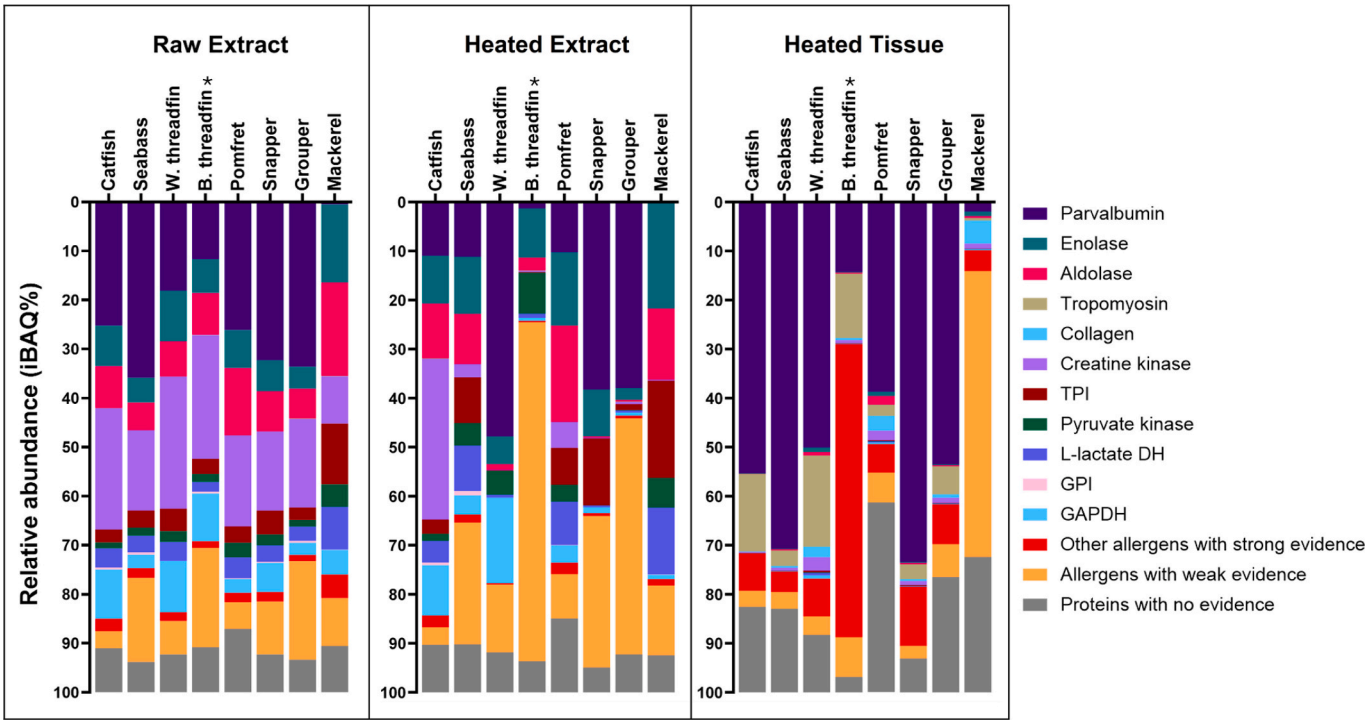


Fig. 6. Allergen profiles across eight fish species under three preparation conditions.
Proteins were classified based on AllerCatPro predictions as having strong, weak, or no evidence of allergenicity in raw extract (RE), heated extract (HE), and heated tissue (HT). Those with strong evidence and matching the 11 fish allergens were grouped accordingly. Note: Missing data points indicate non-detection by mass spectrometry. Asterisk (*) indicates black threadfin, for which interpretation should consider database annotation limitations. TPI, triosephosphate isomerase; DH, dehydrogenase; GPI, glucose-6-phosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

recurrent lower-abundance candidates, including lactoylglutathione lyase, phosphatidylethanolamine-binding protein 1, and superoxide dismutase which were detected in six species, whereas myosin heavy chain, calcium-transporting ATPase, ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 1, like (SERCA-like ATPase), glutamic-oxaloacetic transaminase and 2-iminobutanoate/2-iminopropanoate deaminase, showed more restricted species distributions. White threadfin showed the broadest representation of low-abundance candidates.

Importantly, these proteins should not be interpreted as confirmed allergens, but rather as candidates with significant sequence or structural similarity to known allergen families. Phosphoglycerate kinase and myosin light chain, in particular, were recurrent and quantitatively detectable, making them suitable candidates for prioritisation. Although their allergen status remains mostly predicted by bioinformatic tools, their recurrence across diverse fish species suggests that they conserved components with potential allergenic relevance. However, this does not establish clinical relevance and requires validation through targeted IgE-binding and functional assays to determine whether these proteins contribute to sensitisation or elicitation in fish-allergic individuals. Notably, myosin light chain is recognised as a shellfish allergen [64–66] and was reported as a fish allergen in some cohorts [67], supporting its biological plausibility as an under-recognised or cross-reactive candidate.

Processing further altered the relative contribution of these predicted proteins. In HE, several recurrent candidates, particularly phosphoglycerate kinase and myosin light chain, were generally depleted relative to RE in most species (Fig. 7B). This indicates that a substantial proportion of the candidate fraction is not stably retained in the soluble post-heating extract. Phosphoglycerate kinase, however, showed mild species-specific enrichment in catfish and pomfret. Other proteins that showed depletion in HE includes malate dehydrogenase, myosin heavy chain, SERCA-like ATPase, and glutamic-oxaloacetic transaminase. Nevertheless, some proteins showed relative persistence or mild enrichment in HE, including aspartate aminotransferase, lactoylglutathione lyase, phosphatidylethanolamine-binding protein 1, superoxide dismutase and 2-iminobutanoate/2-iminopropanoate deaminase, although these responses remained species-dependent. Hemoglobin subunit beta, troponin, and myosin heavy chain also showed selected species-specific retention patterns, suggesting that solubility of these candidates post-heating is both protein- and species-dependent.

A contrasting pattern was observed in HT, in which several structural or muscle-associated proteins were preferentially retained in the tissue matrix (Fig. 7C). In particular, myosin light chain showed consistent positive HT/RE shifts across species, whereas troponin and myosin heavy chain exhibited pronounced HT enrichment in the species where they were detected, despite their low raw abundance. 2-iminobutanoate/2-iminopropanoate deaminase also showed HT enrichment in both quantified species (pomfret and mackerel). In addition, superoxide dismutase showed a species-specific reduction in mackerel but relative enrichment in all other quantified species. Conversely, phosphoglycerate kinase and phosphatidylethanolamine-binding protein 1 were consistently depleted relative to RE across all species, consistent with an extract-soluble protein that is poorly retained following tissue heating. These contrasting profiles reinforce the conclusion from the confirmed allergen analysis on soluble and tissue-retained heating effects according to protein biochemical properties and matrix interactions.

Overall, these allergenicity predictions expanded the detectable allergen fractions beyond the currently recognised fish allergenomes and revealed two main patterns: (i) a recurrent component represented by proteins such as phosphoglycerate kinase and myosin light chain, suggesting a conserved background layer of potential allergenic proteins in fish muscle, and (ii) a lower-abundance, species-specific component restricted to selected taxa, with white threadfin showing the broadest representation. This mirrors the broader conclusion that fish allergenicity appears to comprise both shared pan-fish component and species-

(A)

Candidate allergens \ Species	Catfish	Seabass	W. threadfin	B. threadfin	Pomfret	Snapper	Grouper	Mackerel	Detected in X out of 8 species
Phosphoglycerate kinase	0.59%	0.94%	0.72%	0.57%	0.93%	0.95%	0.48%	2.67%	8
Myosin light chain	0.48%	0.09%	0.08%	0.22%	0.09%	0.13%	0.21%	0.13%	8
Aspartate aminotransferase		0.13%	0.17%		0.20%	0.16%	0.06%	0.70%	6
Lactoylglutathione lyase	0.09%		0.15%	0.07%	0.11%	0.10%	0.08%		6
Phosphatidylethanolamine-binding protein 1	0.06%	0.07%	0.06%	0.11%		0.11%	<0.01%		6
Superoxide dismutase	0.03%	0.02%	0.02%		0.02%		0.06%	0.09%	6
Malate dehydrogenase	0.08%		0.13%					0.74%	3
Troponin		ND		0.05%		0.26%			3
Myosin heavy chain			<0.01%	<0.01%				<0.01%	3
Hemoglobin subunit beta	0.85%	0.42%							2
Calcium-transporting ATPase		0.10%			0.21%				2
ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 1, like (SERCA-like ATPase)	0.11%						0.10%		2
Glutamic-oxaloacetic transaminase			0.13%	0.08%					2
2-iminobutanoate/2-iminopropanoate deaminase					<0.01%			0.03%	2

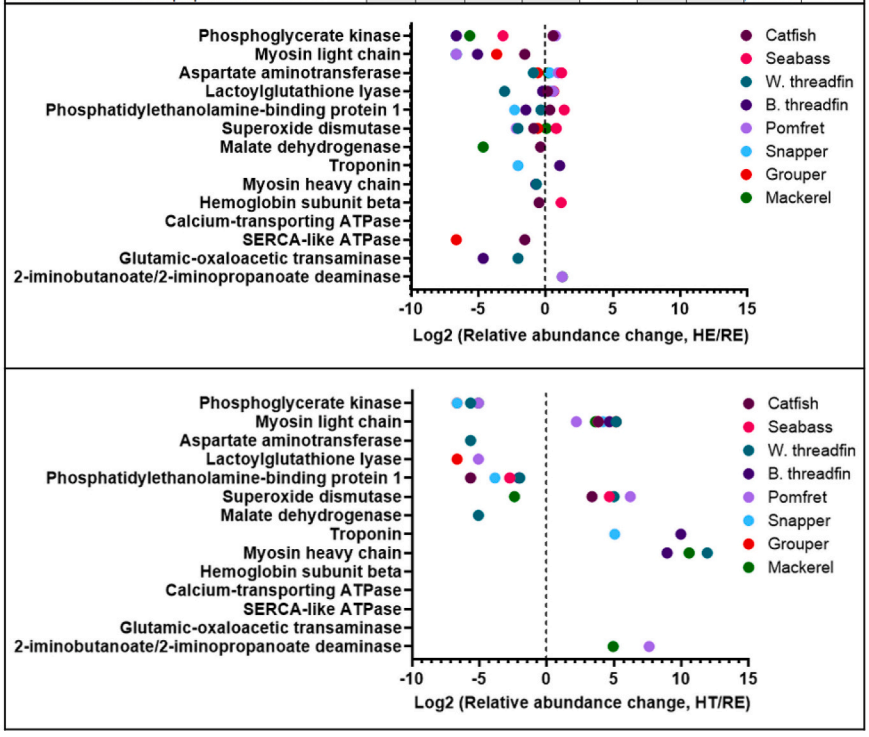


Fig. 7. Prediction-based profiles of candidate allergens across eight fish species.
Relative abundance of 14 recurrent candidate allergens with strong evidence of allergenicity and global iBAQ of >0.05% across all preparation states, (A) identified in raw extracts, together with (B) processing-induced changes expressed as log2 relative abundance change in heated extract/ raw extract (HE/RE) and (C) log2 relative abundance change in heated tissue/ raw extract (HT/RE) ratios. Positive and negative values indicate relative enrichment and depletion in the heated conditions relative to raw extract, respectively. Note: ND indicates not detected or not quantified in raw extract. Missing data points indicate that abundance ratios could not be calculated because the protein was not quantified in one or both paired preparation states.

specific component [35,68].

3.5. Limitations of this study

Several limitations should be considered when interpreting the presented findings. First, quantitative proteomic abundance does not directly correlate to clinical allergenicity, and *in silico* allergenicity predictions remain hypothetical and require further downstream confirmation. Additionally, the small cohort size ($n = 6$) and use of pooled sera immunoblotting do not capture individual sensitisation heterogeneity or estimate the frequency of IgE-reactivity for specific allergens. Further validation using larger, well-characterised cohorts, individual serum, and clinical data will be required for diagnostic recommendations. Second, the study relied on defined extraction and heating workflows that capture important aspects of allergen behaviour but do not fully reflect the diversity of real-world domestic and industrial processing conditions. Insoluble proteins generated through intrinsic physicochemical properties or heat-induced aggregation were removed during clarification, reducing apparent protein recovery, particularly in HT samples. Processing-induced enrichments should therefore be interpreted as relative changes within the extractable fraction rather than absolute increases in total allergen content. Third, limitations in database coverage, taxonomic resolution, and annotation quality restrict protein assignment and allergenicity prediction in some less commonly studied species. While several species were analysed against species-specific proteomes in this study, white and black threadfin required a broader Carangaria *incertae sedis* database and pomfret a family-level (Carangidae) database due to limited species-specific annotation resources. This is particularly apparent in black threadfin, where contrasting trends including an apparent increase in strong and weak evidence fractions after processing (Fig. 6), may partly reflect annotation limitations rather than true biological differences alone. These findings should therefore be interpreted cautiously until supported by improved species-specific databases and comprehensive validation. Furthermore, interpretation of relative iBAQ values should consider the influence of extraction biases, ionisation variability, and

potential batch effects inherent to label-free discovery proteomic workflows. Although identical workflows and analysis parameters were applied across samples, future studies with full biological replication, targeted quantification and batch-randomised acquisition will be required to refine quantitative estimates. Finally, although the eight fish species examined are highly relevant to the Asia-Pacific context, they represent only a subset of the fish species encountered in broader dietary exposure and diagnostic practice.

3.6. Implications on food safety assessments

The present findings have important implications on food allergen monitoring and risk assessment. As processed foods are often assessed using analytical methods developed around raw or soluble protein extracts, the proteins most strongly represented after cooking may differ substantially from those dominant before processing [49]. Quantitative proteomics is therefore well suited for assessing how processing changes the relative prominence of clinically relevant allergen targets [69]. Parvalbumin remained the most consistently represented allergen across species, supporting its continued role as a key marker for fish allergen detection. However, the divergent behaviour of HE and HT also demonstrates that heating a diagnostic or analytical extract is not equivalent to extracting proteins from cooked tissue. Extract-based systems may preferentially recover proteins that remain soluble after heating, whereas consumption of cooked muscle includes exposure to proteins retained within or associated with the tissue matrix. Matrix-dependent recovery should therefore be considered when selecting reference materials and interpreting ELISA- or MS-based allergen measurements. A processing-aware and species-resolved framework is needed for food allergen assessment, as reliance on a single marker or on raw extract assumptions may underrepresent the allergenic complexity of cooked fish foods [3].

3.7. Future directions

Future work should prioritise immunological validation of both established and candidate allergens using larger, well-characterised cohorts of individuals with clinically confirmed IgE-mediated fish allergy. In particular, phosphoglycerate kinase and myosin light chain are recurrent candidate allergens detected in every species in the present study, warranting further investigation through IgE-binding assays, inhibition studies, and functional approaches such as basophil cell activation [70,71]. These experiments will be important to distinguish proteins that are merely homologous to known allergens from those that are clinically relevant. Additional studies should also extend the current processing framework to include a broader range of domestic and industrial processing conditions, food matrices, and preparation methods in order to better capture real-world exposure. Isoform-resolved analysis, epitope-specific characterisation, and integration with clinical readouts such as SPT or component-resolved diagnostics would further strengthen translation of quantitative allergenomics into diagnostic improvement and molecular risk assessment [72,73]. Inclusion of these data will strengthen the utility of proteomics workflow by demonstrating that LC-MS/MS coupled with bioinformatic prediction can move beyond detection of known allergens toward identification of proteins relevant for future molecular risk assessment. Together, these steps would help define both the shared and species-specific components of fish allergenicity with greater clinical and food safety relevance.

4. Conclusion

Highly species-specific fish allergen profiles were resolved, which cannot be adequately represented by a single generic fish allergen profile. Quantitative proteomics identified prominent differences in allergen abundance, isoform distribution and overall proteome composition across eight fish species. Although parvalbumin remained dominant in most species, its abundance varied substantially, while additional allergens also contributed differently across species. Thermal processing demonstrated that heated tissue and heated extract preparations do not produce equivalent allergen profiles. Overall, these findings highlight the value of quantitative proteomics for regulatory and diagnostic applications in aquatic food allergen assessment. By integrating LC-MS/MS proteomics, immunological evidence and *in silico* allergenicity prediction, we established a detailed, scalable workflow for identifying representative diagnostic targets, evaluating processing-dependent allergen exposure, and prioritising candidate allergens for future clinical validation. Beyond allergen characterisation, the resulting dataset provides a foundation for representative diagnostic and regulatory reference materials, seafood traceability, and food processing standardisation in allergen risk assessment.

CRediT authorship contribution statement

Renee Chin: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Janitha Iddagoda:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Sahel Heidari:** Writing – review & editing, Investigation. **Shaymaviswanathan Karnaneedi:** Writing – review & editing, Methodology, Investigation. **Vachiranee Limviphuvadh:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Afifah Sean:** Writing – review & editing, Methodology, Investigation. **Kok Wee Chong:** Writing – review & editing, Conceptualization. **Lynette Liling Tan:** Writing – review & editing, Conceptualization. **Xin Shan Lim:** Writing – review & editing, Methodology, Investigation. **Zhenzhao Li:** Writing – review & editing, Investigation, Formal analysis. **Michael G. Leeming:** Writing – review & editing, Methodology, Formal analysis. **Nicholas A. Williamson:** Writing – review & editing, Methodology. **Sebastian Maurer-Stroh:** Writing – review & editing, Methodology, Conceptualization. **Dianne E. Campbell:** Methodology, Funding acquisition, Conceptualization. **Andreas L. Lopata:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Thimo Ruethers:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

ChatGPT 5.5 (OpenAI) and Claude Opus 4.6 were used to improve the manuscript language and readability. After using these tools, the authors reviewed and edited all content and take full responsibility for the content of the published article.

Funding sources

This work was financially supported by the Good Food Institute (GFI) under its Research Grant Program (Fish tissue proteomics as baseline evaluation for safer cultivated seafood with known allergen profile), the National Research Foundation, Singapore and the Agency for Science, Technology and Research (A*STAR) under the Singapore Food Story (SFS) R&D Programme (W22W3D0003), and the National Health and the Medical Research Council Australia (NHMRC; GNT1086656). Funders played no role in study design, data collection, analysis and interpretation of data, or the writing of this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

J.I. and R.C. are holders of full-time PhD scholarships from James Cook University, Singapore. S.H. holds a full-time PhD scholarship from the Centre for Food and Allergy Research (CFAR) and James Cook University, Australia. S.K. is a CFAR Postdoctoral Researcher. The authors would like to thank Igloo Food and Mr. Melvin for assisting with specimen sourcing and procurements. Biorender was used to create the Graphical Abstract and Fig. 1 (Publication license: https://BioRender.com/vll0l55).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2026.153941.

Data availability

MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD077790. Other related data will be made available on request.

An Integrated Omics Workflow to Resolve Diverse Fish Allergen Profile for Improving Seafood Safety, Allergy Diagnosis and Management (Original data) (PRIDE)

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