

MR THIMO RUETHERS (Orcid ID : 0000-0002-0856-3452)

MR JUAN NIKHIL STEPHEN (Orcid ID : 0000-0002-1923-6932)

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## **Characterisation of Ras k 1 a novel major allergen in Indian mackerel and identification of parvalbumin as the major fish allergen in 33 Asia-Pacific fish species**

Condensed title/running head: Allergens in Asia-Pacific fish

**Thimo Ruethers<sup>1,2,3,4</sup>, Marianne Raith<sup>5</sup>, Michael F. Sharp<sup>1</sup>, Martina Koeberl<sup>6</sup>, Juan N. Stephen<sup>1</sup>, Roni Nugraha<sup>1,3,4</sup>, Thu T. K. Le<sup>1,3,4</sup>, Santiago Quirce<sup>7</sup>, Hong X.M. Nguyen<sup>8</sup>, Sandip D. Kamath<sup>1,2,3,4</sup>, Sam S. Mehr<sup>2,9,10</sup>, Dianne E. Campbell<sup>2,9,11</sup>, Christopher R. Bridges<sup>12</sup>, Aya C. Taki<sup>1,3,4</sup>, Ines Swoboda<sup>5</sup>, Andreas L. Lopata<sup>1,2,3,4</sup>**

<sup>1</sup>Molecular Allergy Research Laboratory, Division of Tropical Health and Medicine, James Cook University, Townsville, Australia;

<sup>2</sup>Centre for Food and Allergy Research, Murdoch Childrens Research Institute, Melbourne, Australia;

<sup>3</sup>Centre for Biodiscovery and Molecular Development of Therapeutics, Australian Institute of Tropical Health and Medicine, James Cook University, Townsville, Australia;

<sup>4</sup>Centre for Sustainable Tropical Fisheries and Aquaculture, Faculty of Science and Engineering, James Cook University, Townsville, Australia;

<sup>5</sup>Molecular Biotechnology Section, FH Campus Wien, University of Applied Sciences, Vienna, Austria;

<sup>6</sup>Technical Development and Innovation Group, National Measurement Institute, Melbourne, Australia;

<sup>7</sup>Department of Allergy, Hospital La Paz Institute for Health Research (IdiPAZ) and CIBER de Enfermedades Respiratorias (CIBERES), Madrid, Spain;

<sup>8</sup>Department of Food Biochemistry, Faculty of Food Science and Technology, Nong Lam University, Ho Chi Minh City, Vietnam;

<sup>9</sup>Department of Allergy and Immunology, Children's Hospital at Westmead, Sydney, Australia;

<sup>10</sup>Department of Allergy and Immunology, Royal Children's Hospital, Melbourne, Australia;

<sup>11</sup>Discipline of Paediatrics and Child Health, University of Sydney, Sydney, Australia

<sup>12</sup>Ecophysiology Group, Institute for Metabolic Physiology, Heinrich Heine University, Duesseldorf, Germany

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**Corresponding author:** Andreas L. Lopata; Pharmacy & Medical Research, Bldg 47, 1 James Cook Drive, James Cook University, Townsville, QLD 4811, Australia; phone: +61(07)47814563; fax: +61(07)47816078; e-mail: andreas.lopat@jcu.edu.au

## Abstract

**Background:** Fish is a well-recognised cause of food allergy and anaphylaxis. The evolutionary and taxonomic diversity of the various consumed fish species poses a challenge in the identification and characterisation of the major fish allergens critical for reliable diagnostics. Globally, fish is a rising cause of food allergy complicated by a large under-investigated variety of species as well as increasing global tourism and trade. This is the first comprehensive study on allergen profiles of heat-processed fish from Vietnam.

**Objective:** The aim of this study was to identify the major heat-stable allergens from frequently exported Asia-Pacific freshwater and marine fish, and characterise the major allergen parvalbumin (PV) from one of the most consumed and exported fish species from Asia, the Indian mackerel (*Rastrelliger kanagurta*).

**Methods:** Heated protein extracts from 33 fish species were separated by gel-electrophoresis. PV isoforms were identified by immunoblotting utilising three different PV-specific monoclonal and polyclonal antibodies and further characterised by mass spectrometry. IgE reactivity was investigated using sera from 21 patients with confirmed fish allergy.

**Results:** Heat stable IgE-reactive PVs, with up to five isoforms per species, were identified in all 33 analysed fish species. In the Indian mackerel, seven PV isoforms were identified by 2D-gel electrophoresis combined with mass spectrometric analyses. The amino acid sequence deduced from cDNA of the most expressed isoform showed a high identity (>90%) to PVs from two other mackerel species.

**Conclusions & Clinical Relevance:** Different PVs were identified as the major heat-stable allergens in all 33 analysed freshwater and marine fish species from Vietnam, many of which are exported worldwide and 21 species that have never been investigated before. The Indian mackerel PV represents a novel fish allergen, now officially registered as Ras k 1. Improved diagnostics for fish allergy against Asia-Pacific species should be developed with focus on PV.

## Introduction

IgE-mediated fish allergy is typically a life-long disease [1], with sensitisation rates of up to 3% in the general population [2], and affecting up to 8% of fish processing workers [3]. A wide range of clinical symptoms, including life-threatening anaphylaxis, can be caused by fish allergens and currently the only treatment is strict avoidance of the implicated fish and

provision of an adrenaline auto-injector [4]. Therefore, the identification and accurate characterisation of fish allergens from distinct species and regions are essential to ensure a correct diagnosis and to facilitate the prevention of allergic reactions.

Fish species vary considerably depending on their geographic location and fish allergic individuals can have restricted sensitisation to a very narrow range of species [5]. The South-east Asian region, including Vietnam, relies heavily on seafood in their diet and Vietnam is the world's fourth largest exporter of fish and fishery products [6], reinforcing the importance of understanding the potential allergenicity of fish from Vietnam. In 2013, the average seafood consumption in Vietnam was 33 kg per capita compared to 22 kg per capita in Europe and Northern America [7]. There are very limited data concerning food allergy in Vietnam. However, a recent population-based survey ( $n=17,659$ ) confirmed that seafood is the most common trigger of food allergy and doctor-diagnosed fish allergy is demonstrated in 1.5% (unpublished data; Ms Thu Le). Previous investigations on allergenicity have focused only on a few fish species exported from Vietnam and other South-east Asian countries, mostly freshwater species including basa fish and Nile tilapia (Table 1).

Parvalbumin (PV) is the major and most characterised fish allergen, reported to account for 70-95% of allergic reactions to fish and fish products [1, 8, 9, 10]. PV is a heat stable intracellular EF-hand calcium-binding protein with a low molecular weight (10-12.5 kDa) and an acidic isoelectric point (pI, 4.1-5.2). Based on biochemical properties, PVs can be divided into the  $\alpha$ - and  $\beta$ -lineages and are present throughout all vertebrates [11-13].  $\beta$ -PVs are highly expressed in bony fish and seem to be the major inducers of IgE-mediated allergic reactions [14].

Since Asia-Pacific fish is predominantly imported frozen and consumed in cooked form, this study focused on identifying and characterising heat stable allergens. Therefore, heat sensitive allergens including aldolase and enolase [15], as well as collagen (being reported to be a common allergen in the Japanese population [16, 17]), were not focus of this study.

The aim of this study was to investigate the IgE cross-reactivity of European fish allergic patients to 16 freshwater and 17 marine fish species frequently exported from the Asia-Pacific region including Vietnam; only ten of these have been previously investigated for allergenic PVs (Table 1). Furthermore, different PV isoforms of Indian mackerel (*Rastrelliger kanagaruta*), a frequently traded species, were characterised in detail and the IgE reactivity demonstrated with Australian patients.

## Methods

### Preparation of fish protein extracts

Fresh muscle tissue from 16 freshwater and 17 marine fish species (Table 1), purchased from markets in Ho Chi Minh City and Vung Tau (Vietnam) was blended with a rotor-stator homogeniser (3 min at 13,000 rpm on ice) in phosphate buffered saline (PBS, 4 mM phosphate; pH 7.1; 3 ml/g tissue). After gentle agitation overnight on ice and subsequent centrifugation (20,000 x g), the supernatant was heated for 5 min in a water bath (95-100°C). Precipitated proteins were removed by centrifugation at 14,220 x g and the heated extracts stored at -20°C.

Indian mackerel was analysed in detail using four different protein extracts. Frozen muscle tissue was homogenised under liquid nitrogen and dissolved in PBS (10 mM phosphate; pH 7.2). For the 'cooked extract', the tissue was cooked in PBS (95-100°C) for 30 min before homogenization. The PBS buffer from the cooking process was analysed as 'boiling buffer'. After gentle agitation overnight at 4°C, precipitated proteins were removed by centrifugation at 52,400 x g. 'Heated protein extract' was produced by heating the 'raw extract' for 30 min at 95-100°C followed by centrifugation at 14,220 x g.

PV from Indian mackerel was purified from the cooked extract by ammonium sulphate precipitation with subsequent dialyses against 100 mM ammonium bicarbonate. The protein concentrations were determined using the Bradford assay (BioRad, USA).

### **Fish allergic patients**

Sera were obtained from 3 European and 18 Australian patients. All included patients had a history of typical IgE-mediated symptoms after ingesting fish, and were sensitive to fish as determined by either ImmunoCAP system (Thermo Fisher Scientific/Phadia, Sweden) and/or by allergen skin prick testing. Patients were recruited from the Medical University of Vienna (Austria), the Hospital La Paz in Madrid (Spain), and the Children's Hospital at Westmead (Australia). Characteristics of the Australian patients are available in the supplement (Table S1). Participants or their parents gave written informed consent and patient anonymity was preserved. Ethical permission was obtained for this study: EK565/2007 and LNR-14/SCHN/185.

### **SDS-PAGE and immunoblotting**

Proteins (5 µg) from all extracts were separated according to their molecular weights using a Mini PROTEAN® SDS-PAGE system (Bio-Rad, USA) as described by Laemmli [18]. Proteins were visualised by Coomassie Brilliant Blue R-250 (CBB) staining.

For the immunoblots, 2 µg of proteins from all extracts were separated by SDS-PAGE and transferred onto nitrocellulose membrane. After blocking for 1 h with skim milk in PBS-T

(PBS with 0.5% Tween-20<sup>®</sup>) at room temperature, the membranes were incubated overnight at 4°C either with monoclonal antibody PARV-19, generated against frog PV (Sigma-Aldrich; diluted 1:3,000), or with polyclonal antibodies raised against PV from Asian seabass (*Lates calcarifer*) (diluted 1:5,000) [19] or from common carp (*Cyprinus carpio*) (diluted 1:10,000) [20] (both generated in-house), or with pooled sera from European patients with confirmed fish allergy (diluted 1:10).

After washing with PBS-T, the membranes were incubated with the corresponding secondary antibody (horseradish peroxidase (HRP)-labelled anti-mouse or anti-rabbit IgG; Jackson Immuno Research Laboratories, Inc. and Vector Laboratories, Inc., respectively; both diluted 1:10,000) for 1 h at room temperature. To detect IgE reactivity from pooled patients' sera, HRP-labelled anti-human IgE antibodies were used (BD Pharmingen<sup>™</sup>; diluted 1:5,000). After subsequent washing, the protein-antibody interaction was visualised using the appropriate substrate (Super Signal<sup>®</sup> West Pico Chemiluminescent substrate by Thermo Scientific and PhosphaGLO<sup>™</sup> phosphatase AP by KPL) and documented with FluorChemE (Biozym Scientific GmbH).

In addition, purified Indian mackerel PV was separated by SDS-PAGE and subsequently immunoblots were performed with the monoclonal antibody PARV-19 (Sigma-Aldrich), in-house generated anti-Asian seabass PV polyclonal antibody [19], and serum from 18 Australian patients with confirmed fish allergy (diluted 1:10). Serum from two non-atopic donors was used as a negative control.

Purified Indian mackerel PV was further analysed by 2D-gel electrophoresis: 40 or 20 µg of PV was first lyophilised and then resuspended in a buffer containing urea (8 M), CHAPS (2%), dithiothreitol (DTT, 50 mM) and Biolyte 3/10 ampholytes (0.2%(w/v)). After overnight incubation, the proteins were subjected to isoelectric focusing using ReadyStrip<sup>™</sup> IPG Strips (11 cm, pH 3-6) in a PROTEAN IEF cell (Bio-Rad, USA) with a maximum current of 50 µA/strip, as per the manufacturer's instructions. The IPG strips were equilibrated with urea (6 M), SDS (2%), glycerol (20%), Tris-HCl (0.375 M; pH 8.8), and initially DTT (2% (w/v)), and subsequently with iodoacetamide (4%). Proteins were then separated by SDS-PAGE. The gel with 40 µg of PV was stained with CBB. Proteins from the gel with 20 µg of PV were transferred onto a nitrocellulose membrane using the Semi-dry TransBlot Apparatus (BioRad, USA) and incubated with a pool of serum from six fish allergic patients (Number 6, 9, and 11-14 in Table S1).

### Mass spectrometry analysis

The most prominent PV bands of extracts from 11 fish species and IgE-reactive bands of purified Indian mackerel PV, separated by 2D-gel electrophoresis, were subjected to mass spectrometric analysis after tryptic digestion (see [21] for details). In brief, the extracted peptides were analysed using an AB SCIEX 5600 qQTOF LC/MS/MS coupled with an ULTRA nanoflow LC (Eksigent, USA). The results were analysed with Mascot daemon search engine and cross-referenced against Uniprot database (April 2017). Variable modifications of carbamidomethyl-C and N-terminus, deamidation N, deamidation Q and oxidation of M were selected.

### **cDNA cloning of PV from Indian mackerel**

RNA was extracted from 100 mg of homogenised Indian mackerel muscle tissue by guanidinium thiocyanate-phenol-chloroform method using TriFast™ (Peqlab). The first cDNA strand was synthesised from 1 µg of RNA with Oligo(dT)19 primers (Microsynth) using a RevertAid Reverse Transcriptase kit (Thermo scientific) following the manufacturer's instructions. For cDNA cloning of the Indian mackerel PV, forward and reverse primers were designed based on the known mRNA-sequence of PV from chub mackerel (*Scomber japonicus*, European Nucleotide Archive (ENA)|BAC66618) and Atlantic mackerel (*S. scombrus*, ENA|FM994926), which are identical in this region, with the addition of a *NdeI* restriction site in the forward primer (forward: 5'-GGTGGTCATATGGCCTTTGCAAGTGTACTGAAAGAT-3', reverse: 5'-GGTGGTTTATCCCTTAATCATGGCTGCAAACCTC-3'). Polymerase chain reaction was performed using reagents from Thermo Scientific (5 min at 95°C; 35 cycles: 30 sec 95°C, 1 min 55°C, 1 min 73°C; 10 min 73°C, ∞ 15°C).

The products were ligated into a pGEM-T Easy Vector (Promega) followed by transformation of XL1 Blue cells. Plasmid DNA was isolated with a GeneJET Plasmid Miniprep Kit (Thermo Scientific) and sequenced by Microsynth AG for subsequent deduction of the amino acid sequence.

### **Sequence alignment and phylogenetic analyses**

The deduced amino acid sequence from Indian mackerel PV was aligned with all known PVs from species of the same fish family (Scombridae) using BLAST (NCBI), with the sequences obtained from the Uniprot database. A molecular phylogenetic tree for PVs from species of the order Perciformes was generated using MEGA 6.6. The molecular relationship of PVs was inferred using the neighbour-joining method. The bootstrap consensus tree inferred from 10,000 replicates was taken to represent the molecular similarity.

## Results

### Parvalbumin represents the major IgE binding allergen in all analysed 33 fish species

Heated protein extracts from 16 freshwater and 17 marine fish species (Table 1) were analysed in this study. All species belong to the group of bony fish (Osteichthyes) and can be classified into 21 families within 7 different orders based on taxonomy (Catalogue of Life, [www.catalogueoflife.org](http://www.catalogueoflife.org)).

For each fish species, the SDS-PAGE profiles of the extracts revealed one or two prominent protein bands of high concentration and up to five additional fainter bands (Figures 1 and 2 A) in the 10 to 13 kDa, molecular weight range of PV. Above 13 kDa, only very faint bands were visible. Protein extracts from freshwater and marine species did not demonstrate any notable differences.

The presence of PV was confirmed by immunoblotting with a monoclonal antibody directed against frog PV and two polyclonal antibodies directed against PVs from Asian seabass and common carp, respectively (Figures 1 and 2 B-D). All three antibodies recognised up to two bands with a strong signal and three bands with a weak signal in each lane, confirming the presence of up to five PVs isoforms in each fish species: for example, in mudskipper (Figure 1, lane 14). Although the number of detected bands and/or the intensity of the signal were different between antibodies and species, the protein patterns of species belonging to the same family were similar.

In addition, a higher molecular weight band at approximately 27 kDa was detected in almost all extracts, when using the anti-carp PV polyclonal antibody (Figures 1 and 2 D). However, this higher band was not detected in the extracts from tinfoil barb, giant snakehead, red tilapia, spotted scar, and yellowtail scad. Instead, a band at approximately 23 kDa was recognised in the tinfoil barb extract. In basa fish extract, the monoclonal antibody PARV-19 detected specific bands above 35 kDa with faint signals at approximately 40 and 55 kDa (data not shown). None of the negative controls showed detectable signals.

The immunoblots with patient sera confirmed IgE antibody reactivity to the major fish allergens between 10 and 13 kDa, identified as PVs by the specific antibodies (Figures 1 and 2 E). No IgE reactivity was detected to bands above 13 kDa. Bombay duck (*Harpadon nehereus*) PV showed the weakest IgE-binding and also the lowest reactivity with all three PV-specific antibodies (Figure 2, lane 18).

Selected most prominent protein bands suspected to be PV after immunoblotting, from 11 commercially most relevant species (clown featherback, basa fish, Chinese pangasid-catfish,

giant snakehead, Indian mackerel, Japanese scad, kissing gourami, largehead hairtail, mudskipper, red tilapia, and yellowtail scad) were confirmed as PV by mass spectrometry (Table S2). In all 18 analysed bands (Figures 1 and 2, highlighted with \*), peptides with homology to a wide variety of known PV sequences were identified.

### **Impact of heating and detailed analyses of PV from Indian mackerel**

The Indian mackerel was the main focus of this study as it is very frequently traded and consumed. Four different protein extracts, with different thermal processing were generated from muscle tissue of the Indian mackerel, separated by SDS-PAGE and further analysed by immunoblotting (Figure 3, **I-V**). The raw extract (**R**) showed proteins between 35 and 70 kDa. Heating (**B**, **C** and **H**) reduced the majority of these proteins seen in the raw extract (**R**). In the heated protein extracts only two higher molecular weight bands were clearly visible at ~40 kDa. In all extracts, the most prominent band was at 11.6 kDa. Antibody binding with all three anti-PV antibodies (**II-IV**) suggests that this band represents PV. Binding of IgE antibody from pooled European patient serum (**V**) demonstrated the IgE reactivity of Indian mackerel PV. Differential antibody binding intensity to the PV band was observed based on the abundance of PV in each extract (heated extract > cooked extract > boiling buffer > raw extract) with all antibodies analysed. Besides PV, no other prominent IgE-reactive protein band was observed. The clinical reactivity of heat-treated muscle tissue was demonstrated by skin prick testing in five of seven patients tested (Table S1).

### **Protein sequence of PV from Indian mackerel**

To determine the amino acid sequence of Indian mackerel PV, cDNA was synthesised for nucleotide sequencing. The sequences of three obtained cDNA clones were identical (Ras k 1 in Table 2 and Figure S1). The deduced 109 amino acid long PV has a theoretical pI of 4.96, suggesting that this PV belongs to the  $\beta$  lineage, which is supported by characteristic amino acids at specific positions (A-14, L-16, C-19, F-67, Q-69, F-79) [22].

The alignment of the novel amino acid sequence of Indian mackerel PV with PVs of four species of the same family (Figure S1) revealed a high identity among the tribe of mackerels (Scombrini). PVs from all three mackerels share at least 93% of their amino acid sequence. In addition, the eight amino acids, which have been predicted as important for IgE-binding in the case of chub mackerel PV [23], are also identical.

The PVs from skipjack and yellowfin tuna, which belong to the tribe of tunas (Thunnini, belonging to the family of Scombridae), have a lower sequence identity of 82% and 71%,

respectively as compared to the Indian mackerel PV. In the two Ca<sup>2+</sup>-binding sites, the amino acid sequences of all PVs are highly conserved.

The molecular phylogenetic analysis of all known PV sequences from species of the order Perciformes, including the family of Scombridae (Figure 4), demonstrates that the Indian mackerel is closely related to Chub and Atlantic mackerel.

### **Multiple Indian mackerel PV isoforms**

Indian mackerel PV was purified for further analyses. The purity was demonstrated by SDS-PAGE (Figure 5 A) and the identity of the 11.6 kDa band as PV monomer confirmed by immunoblotting with PARV-19 and anti-Asian seabass PV polyclonal antibody (Figure 5 B and C, respectively). The latter antibody showed binding additionally to a 23 kDa band faintly visible on the CBB stained gel, presumed to be a PV dimer. Individual IgE reactivity was analysed using sera from 18 fish allergic patients (Figure 5 D). The PV monomer and dimer were detected by 15 and 6 fish allergic patients, respectively, but with varying intensity.

The potential presence and IgE reactivity of different Indian mackerel PV isoforms were further investigated by 2D-gel electrophoresis with subsequent immunoblotting using a pool of the six patients showing binding to the PV dimer (Figure 6). All eight IgE-reactive bands were identified as PVs by mass spectrometry (Table 2). The most abundant PV isoform was 11.6 kDa in size, had an estimated pI of 4.7, and demonstrated the best match to Ras k 1 according to our mass spectrometric analyses. Three additional prominent PV isoforms differed in molecular weight (12.6 kDa) or pI (4.6 and 4.8). The presence of another three PV isoforms with very low abundance was confirmed by mass spectrometry. Identified peptides covered 35% to 89% of the deduced amino acid sequence (Ras k 1).

A stable IgE-reactive PV dimer at 23 kDa was identified by mass spectrometry (Table 2), however, it could not be determined whether different dimers were formed by different isoforms due to the low abundance. The band to signal intensity ratio was higher for the dimer compared to the monomers.

### **Discussion**

Allergy to fish is becoming a more frequent and often life-long trigger of severe allergic reactions, and increasingly reported worldwide. While a cure for fish allergy is not available, accurate and detailed diagnosis is crucial for best patient management. Different fish extracts are commercially available for IgE-based diagnosis, however only about one third are derived

from fish relevant in the Asia-Pacific region. Furthermore, most studies investigating allergic reactions to fish are based on marine fish, while little information is available about freshwater fish and their allergens.

In this study, 16 freshwater and 17 marine Asia-Pacific fish species were analysed for their major heat stable allergens. The vast majority of these species (21) has never been investigated regarding their IgE reactivity before (Table 1). The SDS-PAGE profiles revealed species-specific banding patterns in the molecular weight range of PV (10-13 kDa), with no obvious differences between freshwater and marine fish. The total band intensity was similar for all extracts in this molecular weight range and no prominent bands were visible above 13 kDa. Monomeric PVs were identified in extracts of all 33 investigated species through their binding to PV-specific monoclonal and polyclonal antibodies, combined with mass spectrometric analyses for selected protein bands. All of the identified PVs (up to five isoforms per species) were also detected by IgE antibodies from sera of fish allergic patients', demonstrating their IgE reactivity and the high degree of cross-reactivity between related PVs. Generally, the band intensity on the CBB stained gel often did not correlate with the signal intensity on the immunoblot, probably due to the species-specific affinity of the IgG and IgE antibodies to the PV isoform. This was most prominent for the extract from Bombay duck, suggesting a possible lower allergenicity for this species.

All IgE-binding PV bands were only detected by a combination of all three utilised antibodies, underlying the need of more universal detection methods for this major fish allergen. The commercial antibody PARV-19 raised against PV from frog, has previously been used in different studies and the binding activity in this study seems to be similar [9, 22, 24-26]. The in-house generated polyclonal antibody against Asian seabass PV used in this study detected up to three PV isoforms in extracts from all 33 investigated fish species. Interestingly, the polyclonal antibody generated against carp PV detected, in most fish extracts, not only monomeric PVs between 10 and 13 kDa, but one additional band at approximately 27 kDa consistent with a PV dimer. Binding of this antibody to PV dimers was demonstrated previously [20]. In addition, other possible dimeric forms of specific PVs were identified at 23 kDa in tinfoil barb and Indian mackerel. Previous studies have also reported stable allergenic polymers for example in cod [27], snapper [28], and several other species including pilchard [29]. Generally, about 80% of all well-characterised allergens are forming dimers or oligomers naturally [30]; however, their role in triggering allergic reactions remains unclear and needs further investigations.

It is thought that fish allergic individuals have approximately 50% chance of cross-reactivity to another taxonomically distinct fish species [31, 32]. In contrast, mono-reactivity to only one type of fish is infrequent, but has been reported for example for salmon [33, 34]. The limited cross-reactivity of some patients might be explained by the different IgE-binding epitopes of PVs, which were investigated for five different fish species (Atlantic cod, Atlantic salmon, Baltic cod, chub mackerel, and common carp) [1]. While some IgE epitopes seem to be located in similar protein regions, there are distinct species-specific epitopes for example on salmon PV, possibly explaining the differential antibody response [14].

The molecular phylogenetic tree of amino acid sequences of PVs from fish of the order Perciformes demonstrates the large variety of PV isoforms. PVs of closely related species have mostly a high sequence identity and share one branch of the phylogenetic tree, as shown for Scrombridae species with exemption of PV from skipjack tuna (Kat p 1). In fact, skipjack tuna is a good example of great variances in IgE reactivity within a taxonomically defined fish family [35, 36]. This demonstrated that the taxonomy of fish species needs reconsideration for allergy studies, which is also suggested in other disciplines of science [37]. However, there are also PVs from the same species sharing relatively little of their sequence. The two comprehensively characterised PV isoforms from Asian seabass (Lat c 1.0101 and Lat c 1.0201) for example share only 67% of their amino acid residues, resulting in different IgE antibody-binding patterns [38]. Another example is crimson seabream, whose PVs have only a 72% sequence identity. Overall, this great variance of PVs poses a challenge in the biochemical identification of this major allergen and potential utilisation in component-resolved diagnosis.

This study demonstrates, for the first time, the presence of two allergenic PVs in narrow-barred Spanish mackerel. In contrast, a study by Misnan *et al.* [39] identified a 12 kDa IgE-binding protein-band that was considered to be a minor allergen as only 21.6% of fish allergic patients ( $n=37$ ) showed IgE-binding. The IgE reactivity was not compared with a larger patient cohort, but this PV was strongly detected by all specific antibodies as well as the patient serum pool.

For Japanese scad (*Decapterus maruadsi*) the presence of four allergenic PVs was demonstrated in the present study. Misnan *et al.* [40] described previously four PV isoforms from the closely related Indian scad (*D. russelli*), suggesting a similar IgE reactivity between these two species.

Our present study identified two allergenic isoforms of PV from Nile tilapia and the hybrid with Mozambique tilapia. In contrast, previous studies on allergens from Mozambique and Nile tilapia (*Oreochromis mossambicus* and *O. niloticus*) did not describe PV as an allergen [41-43]. However, the presence of PV was also clearly evident in other studies using immunoassay, DNA application as well as immunoblots [44-46]. Therefore, further investigation to confirm the IgE reactivity and allergenicity of tilapia PV is required.

The Indian mackerel is an important fish commonly consumed in Asia, is exported worldwide, and is the third most captured true mackerel species worldwide. This study identified seven different PV isoforms from Indian mackerel as the major allergens using patient serum IgE and mass spectrometry. As most of the PV isoforms have a very similar molecular weight, the purified allergens were further characterised by 2D-gel electrophoresis and compared to the complete sequence generated using cDNA-based amplicons of the most abundant isoform. Interestingly, all seven PV isoforms differed in their amino acid sequence as well as isoelectric point or molecular weight, however, their IgE reactivity was relatively similar. In contrast, the PV dimer at 23 kDa demonstrated a higher IgE reactivity speculated from the signal intensity to protein ratio. The dimeric or oligomeric formation of allergens with their associated increased allergenicity has been observed for the major cow milk allergen Bos d 5 [30]. The possible impact of dimerisation on the IgE reactivity of PVs is currently further investigated.

Two previous studies on this fish species discounted the importance of low-molecular weight proteins such as PV, and no proteins with a lower molecular weight than 15 kDa were analysed [47-48]. Chatterjee *et al.* [47] found a 23 kDa PV-like low-molecular-weight protein, which could be considered as the identified PV dimer.

The most abundant PV isoform is now registered as Ras k 1 with the World Health Organization and the International Union of Immunological Societies ([www.allergen.org](http://www.allergen.org)) and now the 13<sup>th</sup> fully characterised fish PV registered officially as an allergen. The clinical reactivity was demonstrated by skin prick testing.

The sequence alignment and phylogenetic analysis of this novel allergen implies that patients, sensitised to the Indian mackerel in the tropical regions, will most likely have allergic reactions when consuming the Northern hemisphere mackerels, including Atlantic mackerel (*Scomber scombrus*). In turn this indicates that Northern hemisphere patients with mackerel allergy would react when consuming Indian mackerel. This is of particular importance as by

far the majority of all consumed fish products are processed and distributed worldwide and the exact fish species is often unknown.

The high amino acid sequence identity in the Ca<sup>2+</sup>-binding sites of PVs from mackerels and tunas is likely due to a functional requirement for retaining the metal binding capacity. It was demonstrated that the important amino acid residues for IgE-binding of PV from chub mackerel [23] are identical among different mackerel PVs, but different from tuna PVs. Tuna is often considered as less allergenic [35, 36] due to the low content of PV in different muscle tissues as well as the proposed low allergenicity of tuna PV [49-50]. This might be partially explained by unrevealed conformational-type rather than linear-type IgE epitopes [23, 51] or PV polymerisation [52], as demonstrated here for the Indian mackerel.

In summary, this is the first comprehensive study on the identification of Asia-Pacific fish allergens from Vietnam. It was demonstrated that PV is the major heat stable allergen present in all 33 investigated freshwater and marine fish species, with several isoforms present in most species. Seven PV isoforms from Indian mackerel were identified as the major allergens using patient IgE and sequenced and characterised the major expressed PV (Ras k 1). Exported fish from Vietnam play a major role in the worldwide fish consumption; the European Union imported fishery products with a value of over one billion euros from Vietnam in 2016 alone [53]. Therefore, understanding the potential allergenicity of these fish species is important for improved molecular diagnosis and better patient management worldwide [54]. Consequently, we suggest that diagnostic approaches for patients with fish allergy and analyses of cross-contaminated food products from Vietnam and other Asia-Pacific countries should focus on PV.

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### **Conflicts of interest**

The authors declare that they have no conflicts of interest.

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### Legends to Figures:

#### **Figure 1: Antibody reactivity to heated protein extracts from 16 freshwater fish species.**

Heated protein extracts were separated by SDS-PAGE and either stained with CBB (A) or transferred onto nitrocellulose membranes and analysed with anti-PV monoclonal antibody PARV-19 (B), polyclonal antibodies against PV from Asian seabass (C) or from carp (D) or with a pool of sera from two European fish allergic individuals (E). The common species names and numbers are derived from Table 1. Families in common are labelled with brackets below the species numbers. Prominent PV bands (marked on the right side with \*) were analysed further by mass spectrometry.

#### **Figure 2: Antibody reactivity to heated protein extracts from 17 marine fish species.**

Heated protein extracts were separated by SDS-PAGE and either stained with CBB (A) or transferred onto nitrocellulose membranes and analysed with anti-PV monoclonal antibody PARV-19 (B), polyclonal antibodies against PV from Asian seabass (C) or from carp (D) or with a pool of sera from two European fish allergic individuals (E). The common species names and numbers are derived from Table 1. Families in common are labelled with brackets below the species numbers. Prominent PV bands (marked on the right side with \*) were analysed further by mass spectrometry.

**Figure 3:** SDS-PAGE analysis and **antibody reactivity** to whole protein extracts from **Indian mackerel** (*Rastrelliger kanagurta*).

Boiling buffer (B), raw extracts (R), cooked extracts (C) and heated raw extracts (H) from Indian mackerel were separated by SDS-PAGE followed by CBB-stain (I). Immunoblots were performed with the anti-PV monoclonal antibody PARV-19 (II), polyclonal antibodies against PV from Asian seabass (III), carp (IV), and a pool of sera from three European fish allergic individuals (V).

**Figure 4:** Molecular phylogenetic tree of **amino acid sequences of all known PVs from all fish of the order Perciformes**.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Species of the family Scombridae are shaded. The evolutionary distances were computed using the Poisson correction method. There is a total of 108 positions in the final dataset. Note: The families Carangidae, Cichlidae, Latidae, and Scombridae are considered to belong to Perciformes as per Catalogue of Life ([www.catalogueoflife.org/col/](http://www.catalogueoflife.org/col/)).

**Figure 5:** IgE reactivity of fish allergic patients to **purified Indian mackerel PV**.

Indian mackerel PV was separated by SDS-PAGE and stained with CBB (A). Immunoblots were performed with anti-PV monoclonal antibody PARV-19 (B), polyclonal antibodies to PV from Asian seabass (C) and sera from 18 Australian fish allergic patients (1-18, D) as well as from two non-allergic individuals (N).

**Figure 6:** 2D gel-electrophoresis and **immunoblotting** with a pool of **patient sera**.

Indian mackerel PV was purified and separated by isoelectric focusing followed by SDS-PAGE. Proteins were either stained with CBB (A) or transferred onto nitrocellulose membranes and analysed with a pool of sera from six Australian fish allergic individuals (B). IgE-reactive bands were further analysed by mass spectrometry (number in order of abundance) (A).

**Table 1:** Analysed freshwater and marine **fish species**

Common name	Scientific name	Family
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<b>Freshwater fish</b>	1	Clown featherback	<i>Chitala ornata</i>	Notopteridae
	2	<b>Grass carp</b>	<i>Ctenopharyngodon idella</i>	
	3	<b>Common carp</b> <sup>†</sup>	<i>Cyprinus carpio</i>	Cyprinidae
	4	Tinfoil barb	<i>Barbonymus schwanenfeldii</i>	
	5	Yellowfin Culter	<i>Culter flavipinnis</i>	
	6	Asian red tailed catfish	<i>Hemibagrus wyckioides</i>	Bagridae
	7	Chinese pangasid- catfish	<i>Pangasius krempfi</i>	Pangasiidae
	8	<b>Basa fish</b>	<i>Pangasius bocourti</i>	
	9	<b>Asian seabass</b> <sup>†</sup>	<i>Lates calcarifer</i>	Latidae
	10	Giant snakehead	<i>Channa micropeltes</i>	Channidae
	11	Butterfly peacock bass	<i>Cichla ocellaris</i>	
	12	Red tilapia	<i>Oreochromis sp. - O. mossambicus x O. niloticus</i>	Cichlidae
	13	<b>Nile tilapia</b>	<i>Oreochromis niloticus</i>	
	14	Mudskipper	<i>Pseudapocryptes elongatus</i>	Gobiidae
	15	Kissing gourami	<i>Helostoma temminckii</i>	
	16	Spotted scat	<i>Scatophagus argus</i>	Scatophagidae

Table 1 continued

	Common name	Scientific name	Family	
17	<b>Common sole</b>	<i>Solea solea</i>	Soleidae	
18	Bombay duck	<i>Harpadon nehereus</i>	Synodontidae	
19	Threadfin bream	<i>Nemipterus japonicus</i>	Nemipteridae	
20	<b>Largehead hairtail</b>	<i>Trichiurus lepturus</i>	Trichiuridae	
21	Redspot emperor	<i>Lethrinus lentjan</i>	Lethrinidae	
Marine fish	22	Indian mackerel‡	<i>Rastrelliger kanagurta</i>	
	23	<b>Chub mackerel</b> †	<i>Scomber japonicus</i>	Scombridae
	24	Narrow-barred Spanish mackerel‡	<i>Scomberomorus commerson</i>	
	25	Yellowtail scad	<i>Atule mate</i>	
	26	Japanese scad	<i>Decapterus maruadsi</i>	
	27	Black pomfret	<i>Parastromateus niger</i>	Carangidae
	28	Talang queenfish	<i>Scomberoides commersonnianus</i>	
	29	Torpedo scad	<i>Megalaspis</i>	

*cordyla*

30	<b>Greater amberjack</b>	<i>Seriola dumerili</i>	
31	Black marlin	<i>Istiompax indica</i>	Istiophoridae
32	Unicorn leather-jacket	<i>Aluterus monoceros</i>	Monacanthidae
33	<b>Silver pomfret</b>	<i>Pampus argenteus</i>	Stromateidae

Note: Allergenic parvalbumin (PV) was previously identified in species with bold letters (www.allergome.org). PVs of species with † are registered with the WHO and IUIS (www.allergen.org). Species with ‡ have been investigated for their allergens, but PV was not identified.

**Table 2:** Molecular properties and amino acid sequences of different Indian mackerel PV isoforms. Note: the major PV isoform registered as Ras k 1 is detailed in the last row of the table.

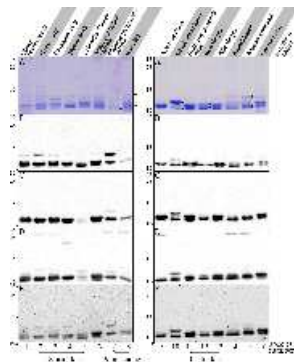
#	MW	pI	Abundance	Sequence fragments of Ras k 1 identified by mass spectrometry	Coverage	Score
1	11.6	4.7	high	-----DAEITAAALDGCKADGSFDHKKFFKACGLAGKSGDDVKKAFAIIDQDK SGFIEEEEELKLFQNFK----TLSDAETKAFKAGD TDGDGKIGVDEFAAMIKG	88%	1860
2	11.6	4.6	medium	-----DAEITAAALDGCKADGSFDHKK---ACGLAGKSGDDVKKAFAIIDQDK SGFIEEEEELKLFQNFKAGARTLSDAETKAFKAGD TDGDGKIGVDEFAAMIKG	89%	686
3	11.6	4.8	medium	-----DAEITAAALDGCKADGSFDHKK-----KAFAIIDQDK SGFIEEEEELKLFQNFK----TLSDAETKAFKAGD TDGDGKIGVDEFAAMIKG	73%	400
4	12.6	4.7	medium	-----DAEITAAALDGCKADGSFDHKKFFK-----SGDDVKKAFAIIDQDK SGFIEEEEELKLFQNFKAGARTLSDAETKAFKAGD TDGDGKIGVDEFAAMIKG	86%	434
5	12.6	4.5	low	-----DAEITAAALDGCK-----KAFAIIDQDK SGFIEEEEELKLFQNFK-----IGVDEFAAMIK-	45%	301
6	10.9	5.0	very low	-----DAEITAAALDGCK-----KAFAIIDQDK SGFIEEEEELKLFQNFK-----	35%	136
7	10.5	4.6	very low	-----DAEITAAALDGCK-----KAFAIIDQDK SGFIEEEEELKLFQNFK-----IGVDEFAAMIK-	45%	386
8	23	4.7	very low	-----DAEITAAALDGCK-----KAFAIIDQDK SGFIEEEEELKLFQNFK-----IGVDEFAAMIK-	45%	405

MAFASVLKDAEITAALDGCKADGSFDHKKFFKACGLAGKSGDDVKKAFAIIDQDK  
SGFIEEEEELKLFLQNFKAGARTLSDAETKAFKAGD TDGDGKIGVDEFAAMIKG

Amino acid sequence of Ras k 1 deduced from cDNA

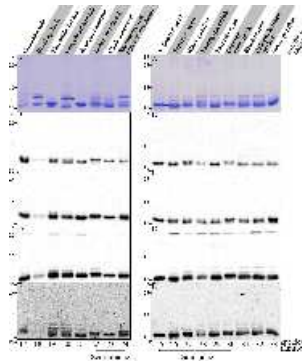
Note: Number in column # refers to spot in Figure 6 A; MW=molecular weight in kDa; pI=isoelectric point; abundance refers to intensity of protein spot in 2D-gel; Score refers to mascot search; '-'=sequence fragment could not be identified by mass spectrometry analyses.

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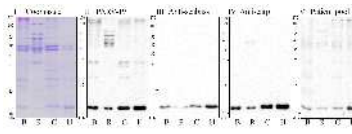
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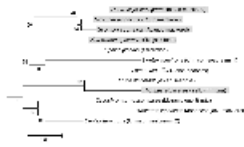
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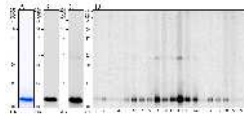
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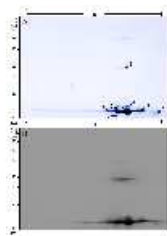
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