The Use of Pangasius Fish in Restaurants

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THE USE OF PANGASIUS FISH IN RESTAURANTS

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Dedicated to

My grandmother Ping Ding and my grandfather Zhijiu Wang.
“The dust returns to the earth as it was, and the spirit returns to God who gave it”,
May you rest in peace as memories of you live on.
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ABSTRACT

Pangasius fish, mainly *Pangasius hypophthalmus* (tra/swai) and *Pangasius bocourti* (basa), which belong to the Pangasiidae family of catfish, are imported farm-raised freshwater fish from Asian. Nowadays, the U.S. is one of the largest importers of Pangasius fish worldwide. As mounting quantities of Pangasius fish being imported every year, the names of “tra/swai” and “basa”, however, seldom appear on restaurant menus. Since processed fish products in restaurants are not subjected to the requirement of labeling species/origins, it is unclear how and to what extent Pangasius are used in restaurants. The overall objective of this study was to investigate if Pangasius fish has been used as a substitute for domestic catfish (Ictaluridae family), high valued fish species, e.g. grouper and snapper, and fish products without specifying the species on the menu from local restaurants.

In total, 47 different fish products from 37 local restaurants in a medium size city were sampled and a commercialized rapid lateral flow strip assay (EZ Pangasius™ kit) were used to identify Pangasius fish. The results showed that 26.7% of domestic catfish tested was substituted by Pangasius, and 22.2% of high-valued fish, grouper, was verified to be Pangasius while no cases of substitution for snapper, sea bass and sole samples tested were found. In addition, a high percentage (66.7%) of dishes displayed under the general name of “fish” on the menu were identified as Pangasius, revealing the favorable choice of imported Pangasius for various fish products due to its sensory quality and cheap price. Isoelectric focusing (IEF) was further conducted to characterize protein patterns of each sample in comparison with authentic fish standards, and the results showed that all the Pangasius positive samples were exclusively tra/swai rather than basa. One year later 7 tested Pangasius positive dishes, including 4 in catfish group and 3 without specifying the species, were sampled again as follow-ups. Indirect non-competitive enzyme linked immunosorbert assay (iELISA), which applied a Pangasius-specific monoclonal antibody (mAb) T7E10, was used to analyze these follow-up samples. The results revealed the continuous use of Pangasius as substitutes for domestic catfish (4 out of 4) and as “anonymous” ingredients for the products without labeling the species. This study exhibited the prevalence of Pangasius in restaurants and also revealed the existence of dishonest behaviors in
restaurant business, which called for the need to set up specific measurements and regulations to discourage the fraudulent practice using tra/swai or basa.
CHAPTER 1

INTRODUCTION

1.1 Pangasius fish

1.1.1 Phylogeny and classification

Pangasius fish is a genus of catfish belongs to the family of Pangasiidae in Siluriformes order, and this family usually inhabits in freshwater in southeastern Asia. The general features of Pangasiidae family are described as laterally compressed body, a separate pair of maxillary and mandibular barbels (no nasal barbels), invariably presented adipose fin and a relatively long anal fin with 26 to 46 rays (Roberts & Vidthayanon, 1991; Teugels, 1996). Species identification as well as systematic classification of the genera within Pangasiidae family have been disputable for decades due to its morphological and ecological diversity. In 1991, Roberts and Vidthayanon proposed two genera according to the morphologic features of Pangasiidae, which encompassed Pangasius Valenciennes, 1840 (19 valid species) and Helicophagus Bleeker, 1858 with two valid species. The key morphological characters used to distinguish fish species among this family contain head shape (e.g., board / narrow, cuspidate / rounded / truncated), mouth shape based on the jaw length (terminal / sub-terminal / inferior), palatal tooth bands, gill rakers, filamentous fin extensions, swim bladder, coloration, vertebral counts, otoliths etc. (Roberts & Vidthayanon, 1991; Teletchea, 2009). New species have been identified continuously and up to date, 28 species are validated within Pangasiidae family (Betancur-R et al., 2013; Gustiano, Teugels, & Pouyaud, 2003; Hee & Kottelat, 2000; Pouyaud, Teugels, & Legendre, 1999).

When focusing on the genus of Pangasius, four subgenera were further recognized, including Pangasius (Neopangasius) Popta, 1904, Pangasius (Pangasianodon) Chevey, 1930, Pangasius (Pteropangasius) Flowler, 1937, and Pangasius (Pangasius) Valenciennes, 1840 (Laurent Pouyaud, Gustiano, & Teugels, 2002; Vidthayanon & Roongthongbaisuree, 1993). Unquestionably, other than traditional phenotypic data such as morphological or physiological traits, taxonomic studies nowadays are enriched by many other disciplines such as phylogenics, systematics and cladistics. The homologous nucleic acid sequences of the macromolecules DNA or RNA in different species are aligned to evaluate the similarities among them. For instance,
Cytochrome b (cytb) is a mitochondrial-encoded gene, which considered to be one of the most useful genes to determine the phylogenetic relationships within genera and family (Farias, Ortí, Sampaio, Schneider, & Meyer, 2001). Based on the analyses of mt cytb sequence as well as allozymes, Pouyaud, Teugels, Gustiano, & Legendre (2000) suggested that the subgenera of P. (Pangasianodon) and P. (Pteropangasius) should be upgraded to generic level while P. (Neopangasius) should be incorporated into P. (Pangasius), which was later supported by alignment of single gene sequences of mitochondrial 12S ribosomal (r) DNA or 16S ribosomal (r) RNA as well as multiple loci analysis which composed of mt cyt b, 12s rRNA, 16s rRNA, valyl-transfer (t) RNA-Val and the nuclear recombination activating gene 1 (rag 1) (Karinthanyakit & Jondeung, 2012; Na-Nakorn et al., 2006; L. Pouyaud, Gustiano, & Teugels, 2004). Although still debatable, the classification of four genera has been embraced by many literatures and databases; and the valid species within each genus of Pangasiidae family were summarized in Table 1 (www.fishbases.org; Ferraris, 2007; Karinthanyakit & Jondeung, 2012; NCBI). Within this taxonomic system, the name of “Pangasius” should refer to the 21 fish species in the genus of Pangasius (Table 1), however, in everyday life, “Pangaius” has been used generally as a collective common name for the imported asian catfish including fish species from P. (Pangasius) as well as other genera, e.g. Pangasius hypophthalmus from P. (Pangasianodon). Since the nomenclature is beyond the scope of our focus, in this paper we use Pangasius as a common name rather than a terminology used in taxonomy.

1.1.2 Habitat and biological characteristics

Pangasius fish distributed throughout south and southeast Asia, which includes India, Bangladesh, Cambodia, Lao People’s Democratic Republic, Thailand, Vietnam, Malaysia, Myanmar (formerly Burma), Indonesia, China and so on (Berra, 2001; Roberts & Vidthayanon, 1991). The geographical distributions of Pangasius actually indicate that it favors warm water (22-26°C) and indeed it cannot endure temperature under 14 °C for extended time due to declined growth rate and diseases-resistant ability (McGee, 2015). Majority of the Pangasius fish are freshwater species with exceptions of P. Pangasius, P. Krempfī, P. kunyit, P. sabahensis and P. mekongensis, which also inhabit in brackish water (Azlina, Daud, Siraj, Aliabadian, & Moghaddam, 2013). Belong to large sized catfishes, Pangasius length varies between 20 cm to 3 m, while most of the fish reach to 50 cm or longer (Roberts & Vidthayanon, 1991).
Pangasianodon genus only contains two species, naming *Pangasianodon gigas* and *Pangasianodon hypophthalmus*, and they distinguished themselves from any other species by having a terminal mouth (upper and lower jaws are nearly equal), a single chambered swim bladder and 8 or 9 pelvic fin rays other than 6 rays. Besides, the mandibular barbels and jaw tooth are gradually disappear when the juveniles grow to the adults (Kakizawa & Meenakarn, 2003; Pouyaud, Gustiano, & Teugels, 2002; Roberts & Vidthayanon, 1991). *P. gigas* is known as Mekong giant catfish and the name implies its native residence in Mekong River and the large size of its body, actually it is one of the world largest freshwater fish which can grow over 3 meters and more than 300 kilograms (Chaijan, Jongjareonrak, Phatcharat, Benjakul, & Rawdkuen, 2010). Unfortunately, because of overfishing and habitat deterioration, *P. gigas* is severely endangered nowadays (IUCN Red List: Critically Endangered A4bcde.), therefore, it processes litter value in the fisheries (Hogan, 2013; Poulsen et al., 2004).

The other species *P. hypophthalmus* (Synonym: *Pangasius hypophthalmus*, *Pangasius sutchi*) is commonly known as tra, swai, Sutchi catfish, striped catfish or Iridescent shark. This species occurs in Chao Phraya River in Thailand and lower Basin of Mekong River (Vidthayanon & Hogan, 2013). Compared with *P. gigas*, tra/swai fish possess smaller body size with maximum length reach to 150 cm, while fish from Mekong delta usually below 50 cm (Poulsen et al., 2004). One unique feature of tra/swai is that it utilize its swim bladder as the accessory respiratory organ and can live from 50 minutes to 18 days without surfacing out of water for oxygen, which means it can endure and survive better in the compromised and deteriorated aquatic environments; therefore, tra/swai is readily adaptive to insensitive farming practices (Browman & Kramer, 1985). Moreover, tra/swai has a rapid growth rate, and the average weight gain ranges between 1.26 g/day to 2.80 g/day based on different feeds; it will only take 6 to 8 months for them reach to commercial weight of 0.8 to 1.3 kg/fish (Ahmed et al., 2013; Sayeed, Hossain, Mistry, & Huq, 2008; VASEP, 2012). At present, this fish species is extensively cultivated and the tra/swai aquaculture has become the industry of important economy pillar of several Asia countries, especially Vietnam.

Speaking of the Vietnam aquaculture, it is inevitable to mention *Pangasius bocourti*, which is another species habitat in Mekong delta basin and dominates the aquaculture sector in Vietnam together with tra/swai. Seeing that they are the most common fish commodities within
Pangasiidae family that imported to the U.S. market, the general morphologic similarities and differences between these two representative species are summarized in details (Table 2); and these two species are the focus of this research paper. The fish of *P. bocourti* belongs to the genus of Pangasius, and basa, bocourti, or simply Pangasius have been used as its common names. Different from tra/swai, the mouth shape of basa is sub-terminal, therefore, the teeth in its upper jaw are still exposed even when the mouth is closed (Table 2). The swim bladder of basa cannot absorb atmospheric oxygen, thus they cannot survive for a long time if there is a low level of oxygen dissolved in the water. In Vietnam, basa is exclusively raised in less intensive cages floating on the rivers while tra/swai is cultured in both cages and ponds (Binh, D'Haese, Speelman, & D'Haese, 2010; VASEP, 2012). Binh *et al.* (2010) reported a significant decline of basa production from 42.2% in 1997 to solely 1.29% in 2005, while tra/swai held 89.3% of the total Mekong delta aquatic production in the same year. The authors attributed this phenomenon to the longer growing period, higher mortality rate, greater production costs associated with basa production and ultimately a lower profit when compared with tra/swai cultivation. Descending production of basa fish can also be supported indirectly by limited availability of basa on the market nowadays.

### 1.1.3 Nutritional values and textural properties

The chemical composition of Pangasius flesh is affected by many factors such as species (Hoque, 2013; Men, Thanh, Hirata, & Yamasaki, 2005), dietary sources (Abdul Kader *et al.*, 2011; Asdari, Aliyu-paiko, Hashim, & Ramachandran, 2011; Liu, Wang, & Ji, 2011), muscle locations (Thammapat, Raviyan, & Siriamornpun, 2010), or even various heat treatment during cooking (Domiszewski, Bienkiewicz, & Plust, 2011). In general, Pangasius is featured with high moisture content up to 80-85 g/100g while protein (12.6-15.6 g/100g) and lipid (1.1-3.0 g/100g) levels are comparatively low (Orban *et al.*, 2008). Lipid content displays variances among species, and basa fish exhibited higher fat deposition rate which was almost as twice as tra/swai (Hung, Suhenda, Slembrourck, Lazard, & Moreau, 2004). Despite the different lipid contents from species to species or portion to portion, the highest lipid composition is always saturated fatty acids (SFA), followed by monounsaturated (MUFA) and polyunsaturated (FUFA) fatty acids (Chaijan *et al.*, 2010; Thammapat *et al.*, 2010). Highly unsaturated fatty acids, in terms of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are targets of interest when
people talk about fish consumption because of their health benefits to decrease the risk of cardiovascular diseases, however, multiple articles showed that Pangasius is not a significant source the omega-3 fatty acids (less than 2% of the total fatty acids content) (Asdari et al., 2011; Thammapat et al., 2010; Usydus, Szlinder-Richert, Adamczyk, & Szatkowska, 2011). On the contrary, Pangasius consists of high quality of proteins which are rich in essential amino acids, in particular isoleucine, leucine, lysine, phenylalanine etc., and it is also a good source for minerals such as potassium, iron, zinc (Ding, Tao, Wei, & Liu, 2011; Men et al., 2005). As a white fish, Pangasius has been accepted by many countries and regions for its favorable characteristics in culinary preparation: juicy, delicate and firm texture, without horizontal bones and lacking of fishy odor (Edgar Chambers & Robel, 1993; Orban et al., 2008).

Nevertheless, the low price, the great accessibility, and its special textural properties brought Pangasius an unexpected “notorious” reputation of being used in decisive practices to replace high-valued fish species, and we will look into this problem of Pangasius in the following two sections.

1.2 Pangasius fraud

1.2.1 Imports of Pangasius in the United States

The cultivation of Pangasius fish, primarily basa and tra/swai, in both cages along Mekong basin as well as in ponds by Vietnamese farmers started from 1960s, and its production has raised dramatically from late 1990s (Phuong & Oanh, 2010). Up to now, Vietnam has established its role as the largest Pangasius exporter worldwide by marketing the fish commodities into almost 150 countries and territories; and in the year of 2014 the total export value ran up to $ 1.8 billion U.S. dollars (FAO, 2014.a). Imports of Pangasius fish into the U.S., mainly in the form of frozen fillet, boomed around 2005 and it has become the fastest growing fish commodity in the U.S. market (Hanson & Sites, 2014; Muhammad, Neal, Hanson, & Jones, 2010 ). In the recent years, U.S. has been recognized as the biggest importer besides the economic body of the European Union (E.U.), and its Pangasius imports from Vietnam kept climbing in 2013 and hit $ 381 million; during the same year Pangasius was ranked sixth among top 10 seafood consumption list of Americans (NFI, 2013; VIETRADE, 2014). The subsequent
year of 2014 witnessed the change of Pangasius market that the two major buyers of the U.S. and E.U. showed sharp declines of Pangasius imports, while mounting imports were observed in Mexico, Asia, Latin America and Saudi Arabia (FAO, 2015.a). From 1st January 2014 to 15th December 2014, the U.S. Pangasius imports valued $ 316.29 million, 13.2% down compared with the same period last year (VASEP, 2015).

1.2.2 Pangasius used as substitutes for other fish species

Pangasius has been frequently found to be associated with the problems of seafood mislabeling or substitution. When initially imported into the U.S., Pangasius was marketed as “catfish” by fish distributors, and numerous reports have shown that Pangasius was used fraudulently as substitutes for U.S. domestic catfish (Ictaluridae family) until FDA ruled that only Ictaluridae family can be labeled and sold as true catfish in the U.S. (FDA, 2002). Moreover its ambiguous vernacular names have been confusions for customers or even fish dealers to identify what they are having, e.g. importers simply call tra or basa “Vietnamese catfish/ Pangasius” in general or frequently misbrand other common names used respectively for tra (swai, Sutchi catfish, striped catfish, Iridescent shark etc.) and basa (bocourti). Due to the traits of fast growing, high yield and low price, imported Pangasius has also become frequent substitutes for wild caught and high-valued fish species, such as grouper, snapper, sole and flounder, who are suffering from limited oceanic resources, long growing period and seasoning harvests (Buck, 2010; Rehbein, 2008; Warner, Timme, Lowell & Stiles, 2012). These deceitful practices have been subject to imprisonment and fines, especially for fraudulently labeled and sold Pangasius as other species in bulk volumes either to avoid paying antidumping duties or for economic gains (NOAA, 2007; U.S. Department of Justice, 2009).

1.2.3 Potential problems associated with species substitution using Pangasius

The first ones to bear the brunt of Pangasius substitution are honest fish distributors/dealers as well as numerous consumers. Given the price disparities between Pangasius and other more expensive fish species, they are paying much more than the values they obtained and the economic losses, sometimes can be disastrous. The average price of Pangasius in the first nine months 2014 was reported to be $ 3.09 per kilogram, which is usually $ 1.5 to $ 2.0 cheaper than U.S. domestic catfish; while the market price for whole grouper fillet
is way high up to $ 24 to $ 28 per kilogram ($ 11 to $ 13 per pound) (FAO 2014.b; Seafood Source, 2014). Potential reasons underneath the price difference between Pangasius and the U.S. domestic catfish were briefly discussed here considering the comparability of these two fish from many perspectives: they are freshwater fish, belonging to the same order of Siluriforme, and they are both farm-raised in ponds. It is self-evident that the cheaper labor in Vietnam has been contributing to the lower price of Pangasius, and it was as low as $ 0.0002 per kg Pangasius for the farmers, $ 0.0007 for the traders and $ 0.0196 for the processors (Loc, Bush, & Khiem, 2010). The other major reason is the inexpensive cost of Pangasius feeds, which have been estimated to account for up to 60% of the total annual operating costs in catfish production, and a higher percentage was reported in Pangasius production (65%-70%) (Buentello, Gatlin Iii, & Neill, 2000; Masser, Woods, & Clary, 2005; Phu & Hein, 2003). Majority of the commercial catfish farming adopt feeds with 28% to 32% crude protein content, and the average price of a typical 32% meal was $ 0.53 per kilogram ($ 483 per ton) in 2013; the fluctuating prices of the major protein source in the meal — soybean as well as the carbohydrate source of the corn were the most possible incentives to changing of the feed cost (Hanson & Sites, 2014; Masser et al., 2005). In the farming practices of Pangasius a decade ago, inexpensive homemade feeds prepared from trash fish, agricultural byproducts such as rice bran and broken rice, and vegetables were used exclusively, and the price was down to $ 0.06 to $ 0.09 per kilogram, which made Pangasius very competitive in the globe market. This circumstance started to alter in 2008 when homemade feeds began to lose its place due to food safety concerns and gradually replaced by commercial pellets; as a consequence, the feed price rocketed to $ 0.48 to $ 0.54 per kilogram (FAO, 2015.b; Loc et al., 2010). The higher investments in feeds might be one cause of the shrinking Pangasius market in the past year or probably affect its production in the long run as well.

Major concerns for Asian aquaculture products have been the abuse or misapplication of various chemicals and antimicrobial agents including parasiticides, fertilizers, disinfectants, antibiotics, either to prevent or treat outbreaks of infectious disease. The use of these chemicals in aquacultured animals raises significant public health and safety concerns. In the U.S. use of antibiotics and chemicals in aquaculture products is illegal under Section 512 of the Federal Food, Drug, and Cosmetic Act (FFDCA), and the presence of chemical residues in seafood
adulterates the seafood under 402(a)(2)(C)(ii) of the FFDCA (U.S. FDA, 2007). Rico *et al.* (2013) reported that antibiotics contributed the highest to the chemical mass inputs of Pangasius, up to 93g per tonne of harvested products. Rather than to prevent disease, these applied antibiotics are more intended to treat the outbreaks of diseases, *e.g.* red spot disease, parasites, and Bacillary necrosis, which are liable to happen with increasing frequency as the intensifying of Pangasius farming practices and deteriorating of the public water quality (Khoi, 2011; Rico *et al*., 2013; Sinh, 2007). It was reported that 15 FDA refusals of imported Pangasius in 2012 were based on the finding of prohibited antibiotics such as nitrofurans, fluoroquinolones, malachite green dye, crystal violet and other chemical pesticides, *e.g.* melamine (Frame, 2013). Negative impacts of aquacultural antimicrobial agents on human health include the development of antibiotic-resistant bacteria and the chronic toxic effects of accumulated chemical residues (Sapkota *et al*., 2008). Dung *et al.* (2008) (Dung *et al*., 2008) studied *Edwardsiella ictaluri* (main cause of Bacillary necrosis) isolated from tra, and concluded a high percentage of resistance to streptomycin, oxytetracycline and trimethoprim. These bacteria with elevated antibiotic-resistance may transfer genetically to the infectious counterparts in human and animal populations.

Another consideration with respect to food safety of consuming farm-raised Pangasius stems from potential metal residues bio-amplified from surrounding aquatic systems. Levels of mercury and lead residues have been shown to exceed the respective maximum residual limits in Pangasius fish (Amin, 2011; Ferrantelli *et al*., 2012). If contaminated fish was served as substitutes to particular individuals, especially women who are pregnant or breast-feeding infants, the consequence can be detrimental if the neonates developed impaired neurological systems or even disabilities. Yet, another group of people might be the victims are the ones suffering from allergic reactions if ingested undisclosed fish species. A case was reported by a British local newspaper that a teacher developed life-threatening allergic symptoms after eating a common fish and chip supper, and the fish was found to be Pangasius later on (The Leicester Mercury, 2011). The majority of patients develop allergic symptoms to fish because of sensitization to a major fish allergen: parvalbumin, which is a low molecular weight muscle protein and is conserved among various fish species. Therefore, patients tend to show cross-sensitization to different fish species; however, clinical cases have reported that patients exhibited allergic
reactions exclusively to pangasius fish while having no problem consuming other common fish species such as cod, salmon, tuna, Pollock, crustaceans and molluscs (Ebo et al., 2010; Raith et al., 2014). If some consumers ordered the grouper dish but turned out to be the Pangasius fish to which they are mono-sensitive, there is a high chance to put their health at risk.

As we mentioned before, Pangasius has been imported into the U.S. primarily in the form of frozen fillets. The absence of morphological characteristics challenges the awareness of the fraudulent practices, and requires the analytical methods to be recruited as powerful tools to discourage seafood adulteration or prevent it from happening. In the third section, the existing detection methods to identify seafood species will be introduced, with a further discussion on their application to Pangasius differentiation.

1.3 Identification of fish species

1.3.1 DNA-based detection methods

Analytical identification of fish species can be classified into two major categories: the deoxyribonucleic acid (DNA)-based (Sriphairoj, Klinbu-nga, Kamonrat, & Na-Nakorn) and the protein-based methods. Having been developed and gained their prevalence during the last two decades, DNA-based techniques, relying on the polymorphisms of DNA among different species (Teletchea, Maudet, & Hänni, 2005). The DNA is extracted from the fish tissue and amplified to millions of copies using polymerase chain reaction (PCR), during which process, either mitochondrial DNA (mtDNA) or nuclear DNA (nDNA) from the fish tissues are the genetic targets of the amplification while the DNA of bacterial communities associated with various fish can also be used to determine the fish origin (Montet, Kouakou, & Le Nguyen, 2012). Subsequently the amplicons are subjected to digestion, electrophoresis or other analytical methods, namely restriction fragment length polymorphism (RFLP), forensically informative nucleotide sequencing (FINS), random amplified polymorphic DNA (RAPD), single-stranded conformational polymorphism (SSCP), amplified fragment length polymorphism (AFLP), real-time PCR (RT-PCR), denaturing gradient gel electrophoresis (DGGE) and DNA chips (Microarrays) (Rasmussen & Morrissey, 2008; Teletchea, 2009). RFLP applies enzymes which can cleave different DNA sequences at the same locus and produce restriction fragments that
varies according species. Thongpan and colleagues (1997) showed Hpa I digestion could result in unique patterns for *P. gigas* (single band of 300 bp), *P. hypophthalmus* (two bands of 100 and 200 bp) and their hybrid (three bands of 100, 200 and 300 bp). Sriphairoj, Klinbu-nga, Kamonrat, & Na-Nakorn (2010) revealed a locus of 204 bp (PL8) could be used as the marker in SSCP analysis to identify the most important commercial Pangasius species including *P. gigas*, *P. hypophthalmus*, *P. bocourti* and *P. larnaudii*; however, this method was not effective in a more complicated situation to differentiate the hybrids of *P. gigas* and *P. hypophthalmus*, *P. bocourti* and *P. hypophthalmus* from their parents. In another study AFLP markers were used successful to identify *P. gigas*, *P. hypophthalmus*, and the first two generations of their hybrids (Sutthi, Amornlerdpisan, Mekchay, & Mengumphan, 2014).

Despite the extensive applications of the DNA-based methods in species identification, they are subjected to several limitations to identify fish species. Generating enough DNA materials is the vital for the succeeding analysis, but DNA is easily denatured due to damages such as heating, low pH, high pressure, drying, irradiation, hydrolysis, enzymatic degradation because of the nucleases, depurination (Teletchea, 2009). Therefore for those processed fish products such as canning, quantities of DNA can be greatly compromised and limiting the downstream analysis. Furthermore, if DNA is contaminated with carryover interspecies genetic materials, which can be replicated to a significant amount during PCR cycles, therefore, leads to false positive results. Another factor could lower the validation of DNA-based methods is the intra-species variations of the DNA sequences which supposed to be conservative for a specific species (Rasmussen & Morrissey, 2008). Finally, for fish species identification, majority of the DNA-based methods rely on the alignments of the sequences in the unknown samples with the existing ones in the DNA databases, which is impossible to do if the target DNA fragments have not been sequenced yet; furthermore, those databases are still being questioned for their reliability for false sequences, missing information, and inconsistent terminology (Rasmussen & Morrissey, 2008; Teletchea, 2009).

1.3.2 Protein-based detection methods

Traditionally, fish species identification has been recruited protein-based techniques such as high-performance liquid chromatography (HPLC) (Armstrong, Leach, & Wyllie, 1992;
Le Fresne, Popova, Le Vacon, & Carton, 2011), isoelectric focusing (IEF) (Hsieh, Chen, & Nur, 1997; Hsieh, Woodward, & Blanco, 1995), capillary electrophoresis (CE) (Dooley, Sage, Clarke, Brown, & Garrett, 2005), two-dimensional electrophoresis (Berrini, Tepedino, Borromeo, & Secchi, 2006; Piñeiro, Barros-Velázquez, Sotelo, Pérez-Martín, & Gallardo, 1998), sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) (Etienne et al., 2000), matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) (Mazzeo et al., 2008) and immunoassays (Chen & Hsieh, 2014; Gajewski, Chen, & Hsieh, 2009). Among these various techniques, IEF and immunoassays are our focus in the paper and will be elaborated below.

1.3.2.1 Isoelectric focusing. IEF is an official method for raw fish species identification (AOAC, 1990). It resolves water soluble proteins at their isoelectric points to form unique band patterns for different fish species. Authentic fish standards are usually analyzed side-by-side with the unknown samples to verify the species. In order to assist the accurate identification of various fish species, the U.S. Food and Drug Administration has compiled an online IEF gel library, which containing gel pattern images for 76 commercial raw fish species sold in the U.S. market (FDA, 2013). Even though processing methods which affect the degree of protein denaturation and insolubilization will change the protein banding profiles, IEF has been successfully applied for species identification of cooked samples when enough thermal-stable muscle proteins were obtained; high concentration of urea (6 - 8M), a typical solubilizing agent in electrophoresis is sometimes used to increase the solubility of the heat denatured proteins by disrupting unwanted hydrogen bonds and therefore the formation of secondary structures, to minimize the interference to the proteins mobility (Etienne et al., 2000; Hsieh, 1998; Hsieh et al., 1997). Rehbein (2008) revealed the unique IEF pattern of P. hypophthalmus, which was different from sole (Solea solea), witch flounder (Glyptocephalus cynoglossus), plaice (Pleuronectes platessa) and catfish (Clarias gariepinus, Siluris glanis), however, limited researches were reported on the application of IEF in identifying various species within the Pangasiidae family, especially the processed fish samples.

1.3.2.2 Immunoassays. Immunoassays have been firstly introduced in 1959 by Yalow and Berson to detect and quantify insulin (Yalow & Berson, 1959). This technique based on the immunological reactions between the antigens and antibodies, and in response to the specific
binding of the antigen and the antibody, detectable labels yield signals which can be then used as a measure to determine target analytes in the samples both qualitatively and quantitatively. The label initially used in the Yalow and Berson’s study was radioactive isotope (radioiodine $^{131}I$) and many non-isotopic forms of the labels have been employed afterwards to avoid potential health hazards. One of the most popular labels that conjugated to the target reagents are enzymes, which can catalyze chromogenic, fluorogenic or chemiluminogenic reactions to generate measurable signals. The most commonly used enzymes are horseradish peroxidase (HRP), alkaline phosphatase (AP) and β-galactosidase ($\beta$-gal), and this type of immunoassays is named enzyme linked immunosorbent assay (ELISA) (Butler, 2000). Over the past half century, diverse designs of immunoassays have been developed such as competitive assays, single-site immunometric and two-site immunometric assays (also known as sandwich assays) (Wild, 2005). Unlike DNA-based and other protein-based techniques which require elaborate analytical instruments, immunoassays can be presented in simple formats such as strips, cassettes or 96-well microtiter plates; meanwhile the operational procedures of immunoassays do not necessarily require skilled analysts and can be completed relatively rapidly, rendering them the most suitable technique for routine sample analysis especially to screen among a large quantity of samples.

Initially designed for endocrinological diagnosis, immunoassays have quickly expanded to many other fields, including determining of fish authenticity, and the most widely applied assay is the ELISA, a kind of enzyme immunoassay, due to its simplicity, specificity and sensitivity (Asensio, González, García, & Martín, 2008). In the early stage of ELISA application, polyclonal antibodies (pAbs) have been employed to differentiate various fish species using the form of indirect ELISA, for instance, among smoked salmon, trout and bream (Carrera et al., 1996), among sole, European plaice, flounder and Greenland halibut (Céspedes et al., 1999), among grouper, wreck fish and nile perch (Asensio et al., 2003.a) and among albacore and other scombrid species (Carrera et al., 2013); however, in those studies, pAbs exhibited strong cross-reactivity with diverse fish species, and additional procedures to block the pAbs with heterologous proteins must be performed to reduce cross-reactivity and obtain species-specific identification. Non-specificity of pAbs originates from their inherent property of containing heterogeneous antibodies which can recognize more than one binding site (i.e. epitope) on the antigen, and cross-reaction is likely to occur if similar epitopes are shared by other antigens in
various fish species. In contrast to pAbs, monoclonal antibodies (mAbs) are homogeneous and react with a single epitope of the antigen, hence have limited cross reactivity with non-specific antigens; nevertheless, given that more than 20,000 fish and seafood species have been estimated for human consumption worldwide, it is extremely challenging to develop mAbs with high specificity without cross-reacting with other seafood species especially the ones closely related (Campbell, 1996; Rasmussen & Morrissey, 2008). Asensio et al. (2003.b) developed a mAb of 1A4 which could discriminate grouper successfully from other 19 fish species, but cross-reacted with wreck fish. Additionally, the high cost at initial stages of mAbs production, requirement of well-trained personnel, and the regulatory constraints limit the availability of mAbs, especially their commercialization (Farid, 2009).

To discourage the substitution of Pangasius for the U.S. domestic catfish, McNulty & Klesius (2005) developed a mAb of 1A10 which could recognize a 36.8 kDa protein from Ictalurus genus (domestic catfish) while did not react with basa protein extracts. It was unknown that if this mAb would cross react with tra/swai fish or not since very limited numbers of fish species (only 7 species) were tested in this study. Moreover, the mAb of 1A10 exhibited low affinity towards the cooked samples (absorbance reading/OD ~ 0.3) when compared with raw fish samples (OD ~ 0.7), suggesting that the target antigenic protein was thermal-labile rather than stable. Actually various processing methods of fish and fishery products, e.g., heating, salting, high pressure of sterilization, smoking, freezing, further challenge the development and validation of the immunoassay as these approaches will alter the fish protein profiles drastically. Take the fish myosin for example that over a temperature range of 30 to 50° C, the hydrophobic and sulfhydryl groups of the myosin increased and heat induced aggregation started to occur; other studies have also shown that myosin would denatured at 100 – 200 MPa and susceptible to denaturation during frozen storage (Angsupanich, & Ledward, 1998; Chan, Gill, & Paulson, 1992; Hastings, Rodger, Park, Matthews, & Anderson, 1985; Reed, Guilford, & Park, 2011). Under those circumstances, the epitopes will possibly be impaired and as a consequence the antigen-antibody interactions are undermined. Therefore antibodies need to be screened against proteins which are immune from changes of the environmental conditions. In compared with the ubiquitous distribution of DNA molecules in all cell types, the proteins profiles are also
subjected to the changes of sample locations, e.g. muscle, bone, age and status, which is another potential limiting factor in the development of immunoassays (Rasmussen & Morrissey, 2008).

In 2009, Gajewski, Chen, & Hsieh successfully developed two mAbs, T7E10 and T1G11, which discriminated the Pangasius fish (both raw and cooked) from more than 70 common finfish and land animals using indirect non-competitive ELISA, and these two mAbs showed no cross-reactivity with any of the non-Pangasius samples tested. The authors also showed that two thermal-stable antigenic proteins (molecular weight of 75 kDa and 36 kDa) were recognized by mAb T7E10 both in basa and tra, while the antigenic protein banding patterns were different for basa (4 bands between 13 to 18 kDa) and tra (2 bands between 15 to 20 kDa) using mAb T1G11. Based on the mAb T1G11 tra and basa could be further differentiated while iELISA was unable to do so. Later on the same group constructed a mAb-based sandwich ELISA (sELISA) to detect the presence of Pangasius fish (Hsieh, Chen, & Gajewske, 2009). Different from the iELISA, the sELISA utilizes a pair of antibodies with one as the capture antibody while the other serves as the detection antibody. The plates are usually pre-coated with the capture antibody which captures target antigen by recognizing a single epitope. Then detection antibody, which is specific to the antigen, is added afterwards to bind to the antigen by detecting different epitopes, therefore, forming “sandwich” structure with the capture and detection antibody resemble two slices of bread and the antigen stuffed in between. The sELISA exhibits high sensitivity and specificity since two structurally different epitopes have to be identified and hence greatly diminish the interference of cross-reactivity (Miller & Levinson, 1996; Paulie, Perlmann, & Perlmann, 2003). For Pangasius detection, a cross reactive mAb F7B8, universally reacts with all fish species, was used as the capture antibody to immobilize the analytes as much as possible, while the biotin conjugated Pangasius specific mAb T7E10, was used as the detection antibody. This assay have been proved to be very sensitive and could detect as low as 0.5% basa or 0.1% tra tissue in cooked crabmeat.

One company named ELISA Technologies, Inc. introduced a commercial test kit (EZ PangasiusTM Kit) based on these two mAbs, however, adopted the format to the lateral flow immunoassays (LFIA), which is more user-friendly especially for the untrained personnel in the fishery industry and can be applied as a powerful tool to rapidly detect Pangasius adulteration for the regulatory bodies. As far as we know, this test kit has been the first and only commercial test
kit for fish species identification. The LFIA is a technique which combines the chromatographic principle and the immunological recognition between different molecules, and it has been estimated that around 40% of the veterinary rapid immunoassay and ELISA tests were performed in the form of LF (Rosen, 2009). A traditional LFIA design usually consists of a sample application pad, a conjugate pad, a reaction matrix and at last an absorbent pad (O’Farrell, 2009). The capture antibody/antigen is immobilized at the conjugate pad while conjugating with colored labels such as colloidal gold (most commonly used) or fluorescent labels. The detection biological components of the assay are presented on the reaction matrix, usually at the test/control line. Initially developed in 1980s, the application of LFIA has expanded to various fields, for example, clinical diagnostics, pharmaceuticals, detection of food and feed materials, veterinary and environmental settings (Posthuma-Trumpie, Korf & Amerongen, 2009). There are many reasons account for its wide acceptance, with the major ones to be the simplicity in this assay design, low cost of production, and ease of use – usually no external reagent and instrument are needed, and the results are easy to be interpreted with minimal education requirement (O’Farrell, 2009). Currently, the majority of the LFIA applications are qualitative and semi-quantitative, while the quantitative analysis is limited.

1.4 Hypotheses and objectives

In the subsection of 1.2.1 of Chapter 1, we have mentioned that as one of the largest importers of Pangasius fish worldwide, the U.S. imports a large quantity of Pangasius every year. However, the names of “tra/swai” and “basa”, seldom appear on restaurant menus, and there are no reports investigating specifically on how Pangasius fish is used in restaurants, where around 70% of seafood consumption in the U.S. is believed to take place (Stier, 2007). There is no standardized seafood labeling legislation and system for restaurants/grocery stores nationwide currently, which might give an advantage for fraudulent practices to occur in the restaurants. Therefore the overall goal of this research is to study if Pangasius is rarely used at restaurant level as indicated by the menus. We hypothesize that 1) Pangasius fish, tra/swai and basa are frequently served as substitutes for domestic catfish or other higher valued fish species, e.g. grouper or snapper in restaurants; and 2) the inferior tra/swai is used predominately considering the higher price of basa fish and its declined production/imports.
In order to test our hypotheses, the specific objectives are as follows:

1) Sampling fish dishes of catfish, other high valued fish species and fish products without specifying the species on the menu from local restaurants, and then identifying the presence of Pangasius fish using the commercial Lateral Flow strip assay kit (EZ Pangasius™); furthermore determining the frequencies of using Pangasius as substitutes for those fish samples in each category;

2) Verifying the LFIA results via iELISA which is using the pangasius-specific mAb T7E10 developed in our laboratory;

3) Revealing the species-specific protein banding patterns of the restaurant samples using IEF;

4) Upon detection of Pangasius fish, the mAb T1G11 was used to further differentiate basa from tra using western blot;

5) Analyze the relationship between the product price and the product authenticity.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and equipment

The Pangasius specific mAbs of T7E10 and T1G11 were previously developed in our laboratory. Sodium hydroxide (NaOH), sodium chloride (NaCl), sodium phosphate dibasic anhydrous (Na$_2$HPO$_4$), sodium phosphate monobasic anhydrous (NaH$_2$PO$_4$), sodium bicarbonate (NaHCO$_3$), sodium carbonate (Na$_2$CO$_3$), trichloroacetic acid (TCA), citric acid monohydrate, sodium dodecyl sulfate (SDS), Tween 20, bovine serum albumin (BSA), Whatman No. 1 and No. 4 filter paper, and 96 well polystyrene microplates (Costar 9018) were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A). Hydrogen peroxide, horseradish peroxidase conjugated goat anti-mouse IgG (Fc specific), 2, 2′-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS), β-mercaptoethanol, phosphoric acid (H$_3$PO$_4$), 5-sulfosalicylic acid dilydrate (C$_7$H$_6$O$_6$S·2H$_2$O) and EZ BlueTM Gel staining were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Coomassie Brilliant Blue G-250, 0.5 M Tris-HCl buffer (pH 6.8), 1.5 M Tris-HCl (pH 8.8), N,N,N,N′-tetramethyl ethylenediamine (TEMED), Precision Plus Protein Kaleidoscope Standards, 30% acrylamide / bis solution, Tris buffer saline (TBS), Tris / glycine buffer, 10 × Tris / glycine / SDS buffer, supported nitrocellulose membrane (0.2 μm), and thick blot paper were from Bio-rad Laboratories Inc. (Hercules, Calif., U.S.A.). Acetic acid was from EMD Serono Inc. (Rockland, M.A., U.S.A.). Anhydrous ethyl alcohol and glycerol were from VWR International, LLC (Radnor, P.A., U.S.A.). Bromophenol blue sodium salt was purchased from Allied Chemical Corporation (N.Y., U.S.A.). Lateral flow strip assay kit (EZ Pangasius™) was ordered from ELISA Technologies Inc. (Gainesville, F.L., U.S.A.). Ampholine™ polyacrylamide gel plate (PAGplate) for analytical isoelectric focusing, pH 3.5-9.5, and broad PI calibration kit, pH 3-10, were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden.). Other materials and equipment used in the paper include knife, cutting board, aluminum foil, sterile sampling bags, Bio-Phoresis Horizontal Electrophoresis Cell (Bio-Rad) connected to a power supply unit (PowerPac 3000, Bio-Rad), refrigerated circulator (HAAKE C25P, Thermo Electron Corporation). All solutions were prepared using distilled deionized pure
water (DD water) from a NANOpure Diamond ultrapure water system (Barnstead Intl., Dubuque, Iowa, U.S.A.). All chemicals and reagents were analytical grade.

2.1.2 Restaurant fish samples and fish standards

A total of 47 samples of fish dishes were collected from 37 restaurants in a medium sized city in Florida from October 2013 to March 2014, and follow-up samples (7 fish dishes from 7 restaurants) were purchased in May 2015. These fish samples were grouped into three types (Table 3). Group A composed of samples which were listed as catfish on restaurant menus; Group B consisted of fish dishes of the species with relatively higher prices, including grouper (Group B.a), snapper (Group B.b), sea bass (Group B.c) and sole (Group B.d); and Group C contained general fish products without specifying the fish species, such as “fish of the day” or “fish platter”. All samples were purchased as take-out orders, and immediately processed for analysis upon receiving. Restaurant samples that were cooked with various sauces and gravies were rapidly rinsed with deionized water and patted dry on a clean paper towel. A portion of lean meat (5 g) cut from each fish sample was obtained for subsequent analysis. All utensils were cleaned thoroughly after handling each sample to avoid cross-contamination. All samples were individually stored in sterile sampling bags under -20 °C until use.

Authentic tra/swai (Pangasius hypophthalmus) and basa (Pangasius bocourti) were provided by the Bureau of Food Laboratory, Florida Department of Agriculture and Consumer Services, and channel catfish (Ictalurus punctatus), black grouper (Mycteroperca bonaci), and red grouper (Epinephelus morio) were purchased from reliable local fish markets and used as fish standards.

2.2 Methods

2.2.1 Lateral flow strip assay

The analysis was performed according to the manufacturer’s instruction. Briefly, after thawing, a portion of 0.5 g fish meat from each sample was placed into one tube containing extraction solution provided by the EZ Pangasius™ Kit. After mixing thoroughly, a test strip was removed from the pouch and placed in the tube of sample extract. After 10 min, the result can be
read through visible color changes at the control line and the test line on the strip. The LFIA strip test kit was operated as immunochromatography, which used a pair of mAbs specific to Pangasius fish. The schematic design of the LFIA strip assay was showed in Figure 1.

2.2.2 Isoelectric focusing (IEF)

Fish was filleted and cut into small pieces (1” x 1”). Raw sample standards were prepared by homogenizing the tissue with DD water at a ratio of 1:2 (w/v). A separate piece of fish meat from each sample was placed in a clean beaker, covered with aluminum foil and then heated in a boiling water bath for 5 min as the cooked fish standards. After cooling, the cooked meat was homogenized with DD water at a ratio of 1:1 (w/v). Restaurant fish samples were prepared and homogenized as the same manner as cooked fish standards. Each homogenate was then centrifuged at 16,000 × g for 3 min, and the clear supernatant was collected for IEF and the soluble protein concentration was determined using Protein Assay kit II (Bio-Rad, Hercules, CA, USA) with BSA as the standard.

Thin-layer IEF was carried out on commercial ampholine PAGplates, pH 3.5-9.5, according to Hsieh (1998) with modifications. The PAGplates were placed on the cooling platform of the Bio-Phoresis horizontal electrophoresis cell, which was connected to a power supply unit. The temperature of the platform was maintained at 10 °C by circulating water through a refrigerated circulator. Each lane 100 µg protein was loaded onto a paper applicator, and 12 µl of broad PI standard, pH 3-10 was loaded as the pI marker. For anode, 1 mol/L H₃PO₄ was used as electrode solution while 1 mol/L NaOH was applied as the cathode solution. The IEF gel was initially run under constant power of 10 watts for 40 min, and then the applicators were removed. IEF was continued at 20 watts for another 40min. The gel was then subjected to fixing for 30 min (29 g TCA and 8.5 g C₇H₆O₆S·2H₂O dissolved in 250 ml dd H₂O), staining for 10 min (0.1% w/v Coomassie Brilliant Blue G-250 in destaining solution), destaining for 3 hrs (ethanol: acetic acid: dd H₂O = 25: 8: 67 % v/v/v) and preserving for 30 min (glycerol: destaining solution =1:9 % v/v). The gel images were analyzed by ChemiDoc TM XRS system with Quantity one software (Bio-Rad, Hercules, CA, USA).
2.2.3 Protein extraction of fish samples

Approximately 5 g of the restaurant fish samples were weighed into a beaker and mashed into particles using a glass rod. Extraction buffer (0.15 M NaCl) was added into the mashed samples (w/v 1:5) and then homogenized at 11000 rpm for 1 min. The homogenized samples were set at 4 °C for 2 hrs before centrifuged at 5000 g for 30 min at 4 °C. The supernatants were filtered through Whatman nr 1 filter paper, aliquoted into the 2 ml micro-tubes and stored under -20 °C until use. Tra/swai and basa standards were prepared via the same way as the restaurant samples, however, before adding the extraction buffer, the raw fish standards (5 g) were added into beakers, covered tightly with aluminum foil and heated in a boiling water bath for 10 min. After being cooled down to room temperature, extraction buffer was added and prepared in the same way as the restaurant samples. The clear protein extracts were used for iELISA analysis as well as SDS-PAGE.

2.2.4 Indirect non-competitive enzyme linked immunosorbent assay (iELISA)

Protein extracts were diluted in 0.06 M carbonate buffer (pH 9.6) to a final concentration of 2 μg/100 μl. The 96-well polystyrene microplate was coated with 100 μl diluted extract per well and then incubated at 37 °C for 60 min. The plate was washed three times with 250 μl per well of PBST (10 mM PBS, pH 7.2 with 0.05 % v/v Tween-20), followed by incubating with 200 μl per well of blocking buffer (1 % w/v BSA in 10 mM PBS) at 37 °C for 60 min. The same washing step was performed twice and then 100 μl primary antibody supernatant T7E10 diluted 1:8 (v/v) in the antibody buffer (1 % BSA in 10 mM PBS containing 0.05 % Tween-20) was added to each well and incubated at 37 °C for 60 min. After washed three times, 100 μl of horseradish peroxidase-conjugated goat antimouse IgG-Fc specific solution (diluted 1:3000 in antibody buffer) was added per well and the plate was incubated at 37 °C for 60 min. Afterwards, the plate was washed five times and 100 μl per well of ABTS was added to develop color at room temperature for 20 min. Reaction was stopped via adding 100 μl/well of 0.2 M citric acid. The absorbance was measured at 415 nm using a microplate reader (MQX200R, BioTek, Winooski, VT, U.S.A).
2.2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

SDS-PAGE was performed according to the procedures of Laemmli (1970) with modifications. For each lane, 8 µg protein in 14 µl extract was loaded onto the stacking gel (5 %, pH 6.8), and then separated on a 14 % SDS-polyacrylamide gel (pH 8.8). The gel was run at 200 V for 45 min by Mini-Protein II electrophoresis system (Bio-Rad) and then stained with EZ BlueTM Gel staining. Following the SDS-PAGE, separated proteins were transferred electrophoretically at 100 V for 1 h from the polyacrylamide gel to a nitrocellulose membrane by a Bio-Rad MiniTrans-Blot unit. The membrane was blocked with 1 % BSA in TBS for 1 hr, and then incubated with the concentrated mAb T1G11 supernatant for 2 hrs at room temperature. Excess amount of mAb was washed away using TBST (TBS with 0.05% Tween-20) and the membrane was blotted with goat anti-mouse IgG-alkaline phosphatase conjugate diluted 1:3000 using antibody buffer for 1 hr at room temperature. The blot was visualized by alkaline phosphatase color reagent A/B (Bio-Rad, 170-6432) and the reaction was stopped by washing the membrane with DD water for 10 min. Precision plus protein kaleidoscope standard was used as molecular weight standard for SDS-PAGE and western blot.

2.2.6 Statistical analysis

Independent samples t-test was used to compare if significant difference in prices was existed between the two populations of Pangasius positive and Pangasius negative samples groups. SPSS software (version 21) was used to analyze data with significance level at $P \leq 0.05$. 

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CHAPTER 3
RESULTS AND DISCUSSION

3.1 Results

3.1.1 Analysis of restaurant samples using EZ Pangasius™ Kit

The general information of the 47 restaurant samples was shown in Table 4. Samples tested in this study represented a variety of fish products including sandwich, finger, basket, platter, sushi roll, salad, taco and wrap etc. Grill and deep frying were the dominating cooking methods of these samples while few samples were either pan fried, blackened, or crusted. Analyzed by the EZ Pangasius™ Kit, a total of 14 samples (29.8%) showed Pangasius positive results among these 47 restaurant fish samples tested (Figure 2). None of these samples were sold as Pangasius fish. The fish samples without notifying fish species (Group C) demonstrated the highest rate of Pangasius (6 out of 9, 66.7%). In the 15 “catfish” samples (Group A), 4 were identified as Pangasius fish instead of domestic catfish (26.7%), while among the 18 grouper dishes (Group B), 4 were recognized as Pangasius (22.3%). No Pangasius fish was identified as a substitute for a small number of snapper, sea bass, and sole samples tested in this study. The LFIA assay was rapid to show clear results within 1 minute, either positive or negative, suggesting that the LFIA assay was very sensitive and rapid to be applied as a powerful tool in the routine analysis of a wide range of raw and cooked products.

3.1.2 Confirmation of the EZ Pangasius™ Kit results using iELISA

In the EZ Pangasius™ Kit, the Pangasius-specific mAb T7E10 was used as the detection antibody. To further confirm the results, we tested several representative restaurant samples from each category using the same mAb T7E10 in the form of iELISA. In total, ten restaurant catfish samples were tested including all four Pangasius positive samples (r8, r32, r35 and r41) and six Pangasius negative samples (r18, r19, r23, r27, r42 and r45). The same number of grouper samples which composed of four Pangasius positive samples (r10, r11, r28 and r29) and six Pangasius negative samples (r2, r7, r9, r21, r43, r46) were also verified by the iELISA. In addition, six Pangasius positive samples (r1, r12, r13, r33, r36, r37) and two negative ones (r34 and r39) from Group C, one snapper (r4) and one sea bass sample were also examined. The
results of the iELISA perfectly matched with the results obtained from the Pangasius Kit that only the Pangasius positive restaurant samples showed strong reactions with mAb T7E10 (OD reading at 415 nm ranged between 2.144 to 2.750), while no signals were detected for all the Pangasius negative restaurant samples using mAb T7E10 (Figure 3.a, b and c).

In order to investigate if the fraudulent practices were continuing nowadays, we re-purchased 7 fish dishes from 7 restaurants as follow-ups one year after our initial study. The samples included 4 catfish dishes which exhibited Pangasius positive results (r8f, r32f, r35f and r41f) and 3 dishes without specifying the species (r1f, r12f and r33f). The samples were tested using the mAb T7E10-based iELISA, and all the 7 samples showed strong reactions with the mAb T7E10 (OD reading between 2.240 to 2.489), therefore verified to be Pangasius fish (Figure 3.d). The results disclosed the continuous use of Pangasius fish in restaurants and reflected that more frequent inspections are needed to improve current situation.

### 3.1.3 IEF protein profiles of the restaurant catfish and grouper samples

In this study, IEF was used to examine the specific patterns of pangasius positive and negative samples. The IEF serves not only as a verification tool for the accuracy of the results obtained from the rapid LFIA strip test, but also to differentiate the species between tra/swai and basa for these Pangasius positive fish samples. Because restaurant fish samples were prepared very differently, both raw and cooked (100 °C, 15 min) standards were included in the analysis. The IEF patterns of authentic Pangasius (tra/swai and basa), and catfish standards together with restaurant “catfish” samples were shown in Figure 4. Cooking seemed to affect basic proteins more than acidic ones with species-specific patterns being concentrated in low pI regions of the gel. Major pI bands for raw catfish standard (rC) were identified at pH 3.40, 4.26, 4.48, 4.88, 5.78 and 7.20, where the first three bands still remained after cooking, thus could be used as representative band pattern for cooked catfish. Raw basa (rB) and tra/swai (rT) exhibited completely different IEF patterns from domestic catfish, however, showed similar banding pattern with each other with the exception of one band. Two distinctive acidic bands retained after cooking, with pIs of 3.80 and 4.01 for basa (cB), and 3.80 and 4.35 for tra/swai (cT). Based on this difference, four Pangasius positive catfish samples (lanes 1 – 4) were all identified as tra/swai, and all of the Pangasius negative restaurant samples exhibited three distinctive bands.
(3.40, 4.26 and 4.48) of authentic catfish. In addition to the three typical catfish bands, an additional band at pH 3.75 was shown in samples at lane 10, 11 and 15 and a band at pH 3.85 in lane 13. These Pangasius negative samples could be related species belong to the same family of Ictaluridae as channel catfish, or their cooking methods or sauces used might slightly altered the overall banding patterns. These species were not further identified due to beyond the main focus of the present research.

For restaurant “grouper” samples, all of the four Pangasius positive samples showed two distinctive bands (pHs 3.80 and 4.35) of the tra/swai standard, therefore were positively identified as tra/swai (Figure 5). Only one major and distinctive acidic band (pH ~ 4.25) were obtained for authentic black and red grouper standards after cooking. The banding profiles in samples on lanes 5 – 8 were different from the typical grouper patterns, indicating other grouper species or other non-grouper species being possibly used. All other restaurant “grouper” samples showed the band matched to the cooked black or red grouper standards (data not shown). In such case, an overall higher violative rate by including substituting species tested via Pangasius kit (22.2%) could be expected. Authenticity identification of grouper samples was of greater complexity since various inexpensive fish species, such as Pangasius, weakfish, king mackerel etc. have been reported as substitutes for grouper (Warner et al., 2012).

3.1.4 Analysis of the restaurant samples using SDS-PAGE and Western blot

The SDS-PAGE profiles of the restaurant Pangasius positive samples were shown in Figure 6. The major bands of the raw basa and tra standards were clustered between 36 kDa and 60 kDa, and most of them diminished drastically after cooking at 100 °C for 10 min. In contrast, the bands of a 36 kDa protein as well as two small ones (11 and 13kDa) became intensified after heating, suggesting that these proteins were heat stable. In addition, two protein bands around 15 and 20 kDa appeared after the heat treatment and they might be the denatured products of the weakened bands (Figure 6.a). For the cooked basa and tra standards, five most prominent bands were recognized, among which three were shared by both of the species (11kDa, 15kDa and 36kDa) while molecular weight of one protein around 13 kDa in basa fish was slightly higher than tra, and another one of 20kDa was lower than tra. It could be seen that the catfish standard as well as one restaurant Pangasius negative sample (r20) also shared the same bands (11kDa,
15kDa and 36kDa) with the Pangasius fish and meanwhile showed different patterns at 13 kDa and 20 kDa, suggesting that those two proteins might provide species-specific information. Although repeatable results were obtained for these samples, the differences of the SDS-PAGE band pattern were too subtle to be used as a reliable judgment to discriminate basa from tra. Furthermore, the correct interpretation of the results was easily subjected to operational mistakes, which led to the skewed or distorted gel and made it difficult to detect the minor differences.

As shown in the previous study of our group that the western blot revealed different characteristic antigenic protein banding patterns for basa and tra/swai by the mAb T1G11 (Gajewski, Chen, & Hsieh, 2009). Two protein bands were recognized for tra/swai between 15 to 20 kDa while four bands showed up for basa with molecular weight ranged between 13 and 18 kDa. This finding enabled further discrimination of basa and tra/swai, therefore, was employed in this study to validate the results we obtained from IEF. The basa and tra/swai standards showed similar banding patterns as in their paper, however, one additional major band around 30 kDa showed up and this protein was recognized in all the restaurant samples as well (Figure 7). Two types of banding patterns were observed for the Pangasius positive samples, among which three (r28, r29 and r32) exhibited the same pattern as the tra/swai standard (two major bands between 15 to 20 kDa) and therefore were positively verified to be tra/swai. For the rest five samples (r10, r11, r8, r35 and r41), an extra protein with molecular weight around 15 kDa was recognized by the mAb T1G11. Some minor bands observed for the restaurant samples probably resulted from incomplete denaturation, which subjected to the difference in cooking methods and time in different restaurants.

3.1.5 Analysis of sample prices

The average price of all the fish samples tested was $10.61, with the lowest and highest value to be $ 3.19 (r34 fish sandwich) and $ 23 (r17 tasty red snapper), respectively. Among the three categories, the mean price of the grouper dishes was the highest ($ 11.95) and 15 out of the 18 grouper samples cost more than $ 11 while the other three lowered the mean price were dishes served with limited amount of fish flesh, including r7 grouper finger for kids ($ 4.00), r6 sushi roll ($ 6.00) and r9 grouper salad ($ 8.99). For the catfish dish samples, the average price was $ 9.80 and the majority of the samples cost $ 9 or higher, while r41 was an exception which
was provided as a lunch item in a local buffet restaurant ($5.38). The lowest mean price was observed for fish dishes without specifying the species ($7.19). Based on our investigation, Pangasius fish was used for high-priced fish dishes as well as cheaper ones. The four Pangasius positive grouper dishes was sold at an average price up to $14.97, while the lowest price of Pangasius positive sample was $5.38. The results suggested that Pangasius fish has been used either for extra economic gains or to provide competitive low prices to attract more customs.

Mean price of Pangasius negative and positive groups was $10.74 and $10.31 respectively. Frequency distributions of these two groups were shown in Figure 8. Median price of Pangasius negative samples was $10.50, which almost equaled to the mean price, while 50% of the prices in Pangasius positive group was clustered below $8.99 (median). Interquartile range (IQR) of negative sample prices (between $8.99 and $12.50) was more narrowly distributed than positive group (between $6.97 and $14.95), implying the latter group has lower consistence of the prices. The result of independent sample t-test showed that no significant difference in price means could be found between Pangasius negative and positive samples. Therefore, the price could not be a good indicator for the fish authenticity. A sense of ethical responsibility might play a more important role rather than the pricing.

3.2 Discussion

High percentage of Pangasius in Group C indicated the preference and prevalence of this inexpensive fish in restaurants, in order to provide price-favorable products while saving their effort to disclose fish information to customers. Actually, a high ratio of the fish dishes in Group C was told to be “grouper” by restaurant servers or managers when the authors asked what the fish was, which implied that either they were unaware of the fish species they were serving, or they intended to mask their products. It was difficult to determine if such behaviors constituted fish fraud since the dishes were only labeled generally, e.g. “fish sandwich”, “fish platter”, instead of specifying what they were on the menus or on the receipts. In another occasion it is even harder for the consumers to be aware of what they are eating, when fish sauce is added for flavor or smashed fish flesh is used as an ingredient for other food entrees.
Grouper fish belong to the Serranidae family and they are economically important tropical and subtropical fish naturally occur around coral and rocky reefs. The most commonly seen groupers are from the two major genera of *Epinephelus* and *Mycteroperca* (Sumathi *et al*., 2015). Groupers are high valued food fish popularized by their nutritional values, white flesh with large flakes, firm texture and good taste with mild and subtle flavor; but many species have been greatly endangered due to over-fishing. The long sexual maturation time, long-lived adults, and the formation of spawning aggregations make them more vulnerable to fishing practices (Craig, Sadovy & Heemstra, 2011; Pierre *et al*., 2008; Stile *et al*., 2013). The higher market price of grouper usually makes it a target of substitution with less expensive species, for instance, hake, tilapia, channel catfish, alaska pollock, mackerel, perch, pangasius and whitefin weakfish; and it is even more problematic in Florida where around 85% of the nation’s groupers are harvested and supplied (Florida Department of Agriculture and Consumer Services, 2013; Jacquet & Pauly, 2008; Stile *et al*., 2013). The premium economic incentive drives the fraudulent substitution. In a local supermarket, the frozen grouper fillet costs $ 15 per 12 ounce while tra/swai fillet in equivalent amount is only sold at $ 4, which means the distributors or sellers can profit hundreds times more from marketing Pangasius as grouper. Numerous news and investigation reports could be found about masquerading cheaper fish species as grouper both in bulk volumes and retail level. One California Corporation, Seafood Solutions Inc. was sentenced to pay $ 1 million in fines for misleadingly labeled tra/swai as “Paradise Grouper” and “Falcon Baie Grouper”, and around $ 2 million worth of tra/swai fillets as grouper were sold between February 2006 to April 2006 (U.S. Department of Justice, 2012). At retail level, the percentage of mislabeled grouper was up to 40 to 50% in the middle-2000s while more recent studies by OCEANA showed 26% of mislabeled grouper in grocery and a higher ratio in restaurants (31%) (Warner *et al*., 2012; Warner, Timme, Lowell & Hirshfield, 2013). Our study revealed that 22.3% of the grouper dishes were replaced by Pangasius in restaurants, however, the ratio could be higher given that other fish species were possibly be used. Generally, the grouper fillet is thicker than the Pangasius fish, and grouper usually depart in large flakes/chunks and the texture is firmer. The consumers should raise their suspicion if they are served with thin fish fillet which crumble easily into small pieces.
Although FDA ruled out that Pangasius fish could no longer be sold and labeled as “catfish” in the U.S., transmission from law to our plates seemed not very satisfactory. In 2011, an investigation showed that 14.3% (3 out of 21) of catfish products which purchased from retail stores and restaurants in the areas of New York, New Jersey and Connecticut were Pangasius fish rather than domestic catfish (Consumer Reports Magazine, 2011). It appeared that this situation has not been improved because our current investigation reported a 26.7% substitution of domestic catfish using swai/basa in restaurants. Catfish belongs to the same order of Siluriformes as Pangasius, and they share similar textural properties with each other. The sensory assessment studies exhibited textural resemblances of cooked channel catfish and tra/swai fillets, which including juiciness, flakiness, gelatinousness, low firmness and fibrousness (Edgar Chambers & Robel, 1993; Phan & Nguyen, 2012). Unlike commonly consumed meat products like beef, pork and poultry, the similar textural features and numerous varieties of preparation make it difficult to differentiate subtle differences between similar fish species, especially for inexperienced consumers. Moreover, our investigation showed that all of the cooked catfish samples (100%) were coated with a mixture of flour/spices and then deep fried, which concealed the morphological features of fish and imposed greater difficulty on customers’ identification. Cooking methods could be utilized by restaurants to mask the practices of fish substitution or adulteration.

Revealed by the IEF results, a surprising finding in this study was that the exclusive use of tra/swai as Pangasius substitutes for restaurant catfish or grouper dishes, rather than basa. Tra/swai grows faster than basa and is cheaper to be cultured. Tra/swai only takes 6 to 8 months growing time to be commercially available comparing with basa which requires 10 to 13 months. In addition, they can tolerate severe environmental condition, e.g. water with low level dissolved oxygen, and basa is whiter and thicker in flesh and more delicate in texture than tra/swai which has a coarse texture (U.S. ITC, 2003). Therefore basa is considered to be superior to tra/swai regarding the taste and texture. It seemed that better product attributes did not make basa more prevalent on the market. In contrast 95% of U.S. Pangasius imports were constituted with tra/swai, which probably benefited from its greater availability and associated lower price. The culturing method for Pangasius has been shifted from floating cage to farming pond from 2005 in order to meet the strict hygiene and safety criteria in the international markets, therefore,
tra/swai is much more cultivated due to its physiological advantages (Friedrick, 2009; VASEP, 2012). Our finding was consistent with the high imports of tra/swai and showed a high acceptance to use tra/swai as a source of whitefish in U.S. restaurants. It is worth mentioning that previous reports indicated basa has been a major substituting species for grouper, and there was a high possibility that basa displayed as “grouper” were further substituted by inferior and cheaper tra/swai given the high proportion of the tra/swai imports (Edwards, 2007; Abelson & Daley, 2011). These could be attributed to universal unfamiliarity of the differences between basa and tra/swai when they were initially imported into the U.S.

The western blot results showed that mAb T1G11 recognized an extra protein at 15kDa for five restaurant samples (r10, r11, r8, r35 and r41), when compared with the tra/swai standard. As a consequence, we could only conclude that those five samples were not basa fish since the obvious dissimilarity of the banding pattern from basa standard, while asserting them to be tra/swai would be uncertain due to the appearance of the additional band. Many research studies have been reported on the application of the hybrids between different species in aquaculture to obtain optimal growth performance/production or lower pressure for endangered species (Bartley, Rana & Immink, 2000). As an important economic fish species in the aquaculture sector of the countries along Mekong river, the genetic resources of tra/swai have been explored extensively through selective breeding and hybridization (Nguyen, 2009). The available hybrids were *Pangasianodon hypophthalmus* (Sauvage, 1878) × *Pangasius bocourti* (Sauvage, 1880), and *Pangasianodon hypophthalmus* (Sauvage, 1878) × *Pangasius djambal* (Bleeker, 1846), however, as Nguyen (2009) specified in the paper that, the hybridization were only practiced in Thailand and Indonesia rather than Vietnam. It is unclear that the novel band pattern of the restaurant Pangasius samples could possibly be caused by the hybrids circulating on the market. No further effort in this study was made on this question since it involved the farming practices of tra/swai, which was way off the focus of our study.

In 2013 imported catfish-like frozen fillet, primarily Pangasius, accounted for 80% of the U.S. catfish market, which was four times compared with 2005 (Hanson & Sites, 2014). As the market share changes dramatically, disputes have been arisen and still going on as a hot topic that if catfish should go through more rigorous inspections to insure safety and health of the fish products regardless of their resources. At present, it seems that policies are tightening up that the
U.S. Department of Agriculture (USDA) catfish inspection program, which was authorized in 2008 farm bill, would be continued in 2014 farm bill despite many arguments (Flynn, 2014). Also, in this year as one of the top catfish growers in the U.S., Alabama proposed one act which required all the restaurants in Alabama to provide the fish name of Siluriformes order on the menus (Alabama Senate Bill-335, 2014). Nevertheless, it is difficult to foresee the effectiveness of these attempts in short-term. Studies which applied different statistical models showed consistent result that relatively higher price of domestic catfish was the incentive force to boost catfish fillets imports (Ligeon, Jolly & Jackson, 1996; Quagrainie & Engle, 2002; Muhammad & Jones, 2009). Even though U.S. International Trade Commission (U.S.ITC) imposed penal duty of anti-dumping on Vietnam-the largest Pangasius exporter globally and raised tariffs on frozen tra/basa fillets up to 64%, modest benefits for domestic catfish industry were anticipated due to the remaining competitive price of Pangasius (U.S.ITC, 2003; Muhammad, Neal, Hanson, & Jones; 2010). It is most likely for restaurateurs continue to purchase cheaper imported Pangasius to avoid paying premium for domestic catfish products.

Currently there is no standardized seafood labeling legislation and system for restaurants/grocery stores nationwide, but it seemed that different local authorities have realized the significance of this issue and responded in a legislative manner lately. In February 2014, Maryland Seafood Authenticity and Enforcement Act was introduced and the bill required lawfully that food fish or shellfish could not be sold unless being identified the species in a certain manner (on a label, sign or menu) at the point of sale (House Bill 913, 2014). At the same time period, similar act was also introduced independently in California and passed in Senate in August, with a requirement of clear display of species for the fish/shellfish offered for sale (Senate Bill-1138, 2014). These reinforced legislations are believed of great importance to provide better information to customers, and play more powerful roles to fight fish fraud.

3.3 Conclusions

This study investigated specifically how imported Pangasius fish was used in U.S. restaurants. The restaurant samples were tested using both commercial EZ Pangasius™ Kit and Pangasius specific mAb T7E10-based iELISA. The results revealed fraudulent practices of using Pangasius to substitute domestic catfish (26.7%) and high-valued fish species grouper (22.2%).
As retailers and restaurant owners illegally sell the inexpensive fish species at a higher value to make premium profits, the most direct consequence of this deceptive practice is the economic losses of the consumers, especially the ones dining out a lot. Health concern is another important reason that the consumers need to be aware of what they are eating is the substitute fish might contain residues of antibiotics, heavy metal, and allergens they are trying to avoid due to health conditions. A high percentage of Pangasius (66.7%) was used for products without specifying fish species, suggesting the preferable role of Pangasius in restaurants due to its cheap price. Therefore, in order to discourage the fraudulent practice using tra/swai or basa, specific measurements, in particular labeling regulations, need to be tightened up to improve the traceability of fish products along supply chains as well as on the restaurant menus by indicating the species, country of origin and other relevant information, which would be helpful for consumers to know what they have paid for. Follow-ups of 7 restaurants showed persistent use of Pangasius (100 %), which reflected the fact that more frequent inspections are needed to improve current situation.

Restaurant Pangasius-positive samples were verified exclusively as tra/swai rather than basa in this study using the IEF, however, the western blot results revealed the possible existence of the Pangasius hybrids on the market. At last, no significant difference in price means could be found between Pangasius negative and positive samples. Therefore, the price could not be a good indicator for the fish authenticity.
# APPENDIX A

## TABLES

Table 1. Summary of the genera and species in Pangasiidae family. In total, 28 species in four genera are included. *Pseudolais is used as the senior synonym for Peteropangasius.

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>Common name(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pangasiidae</td>
<td>Helicophagus</td>
<td><em>Helicophagus leptorhynchus</em> (Ng &amp; Kottelat, 2000)</td>
<td></td>
</tr>
<tr>
<td>(Bleeker, 1858)</td>
<td>(Bleeker, 1858)</td>
<td><em>Helicophagus typus</em> (Bleeker, 1858)</td>
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<tr>
<td></td>
<td></td>
<td><em>Helicophagus waandersii</em> (Bleeker, 1858)</td>
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<tr>
<td></td>
<td>Pangasianodon</td>
<td><em>Pangasianodon gigas</em> (Chevey, 1931)</td>
<td>Mekong giant catfish</td>
</tr>
<tr>
<td>(Chevey, 1931)</td>
<td></td>
<td><em>Pangasianodon hypophthalmus</em> (Sauvage, 1878)</td>
<td>Tra, swai, striped/suthi/shar k catfish</td>
</tr>
<tr>
<td>*Pseudolais</td>
<td>(Vaillant, 1902)</td>
<td><em>Pseudolais micronemus</em> (Bleeker, 1847)</td>
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<tr>
<td></td>
<td></td>
<td><em>Pseudolais pleurotaenia</em> (Sauvage, 1878)</td>
<td></td>
</tr>
<tr>
<td>Pangasius</td>
<td>(Valenciennes, 1840)</td>
<td><em>Pangasius bocourti</em> (Sauvage, 1880)</td>
<td>Basa</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pangasius conchophillus</em> (Roberts &amp; Vidthayanon, 1991)</td>
<td></td>
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<td></td>
<td></td>
<td><em>Pangasius djambil</em> (Bleeker, 1846)</td>
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<td></td>
<td></td>
<td><em>Pangasius elongatus</em> (Pouyaud, Gustiano &amp; Teugels, 2002)</td>
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<td></td>
<td></td>
<td><em>Pangasius humeralis</em> (Roberts, 1989)</td>
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<td></td>
<td><em>Pangasius kinabatanganensis</em> (Roberts &amp; Vidthayanon, 1991)</td>
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<td></td>
<td></td>
<td><em>Pangasius krempfi</em> (Fang &amp; Chaux, 1949)</td>
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<td></td>
<td></td>
<td><em>Pangasius kunyi</em> (Pouyaud, Teugels &amp; Legendre, 1999)</td>
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<tr>
<td></td>
<td></td>
<td><em>Pangasius larnaudii</em> (Bocourt, 1866)</td>
<td>Spot pangasius</td>
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<tr>
<td></td>
<td></td>
<td><em>Pangasius lithostoma</em> (Roberts, 1989)</td>
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<tr>
<td></td>
<td></td>
<td><em>Pangasius macronema</em> (Bleeker, 1851)</td>
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<tr>
<td></td>
<td></td>
<td><em>Pangasius mahakamensis</em> (Pouyaud, Gustiano &amp; Teugels, 2002)</td>
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<tr>
<td></td>
<td></td>
<td><em>Pangasius mekongensis</em> (Gustiano, Teugels &amp; Pouyaud, 2003)</td>
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<tr>
<td></td>
<td></td>
<td><em>Pangasius myanmar</em> (Roberts &amp; Vidthayanon, 1991)</td>
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<tr>
<td></td>
<td></td>
<td><em>Pangasius nasutus</em> (Bleeker, 1863)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td><em>Pangasius nieuwenhuisii</em> (Popta, 1904)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td><em>Pangasius pampus</em> (Hamilton, 1822)</td>
<td>Yellowtail catfish</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pangasius polynyanodon</em> (Bleeker, 1852)</td>
<td></td>
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<td></td>
<td></td>
<td><em>Pangasius rheophilus</em> (Pouyaud &amp; Teugels, 2000)</td>
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<tr>
<td></td>
<td></td>
<td><em>Pangasius sabahensis</em> (Gustiano, Teugels &amp; Pouyaud, 2003)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pangasius sanitwongsei</em> (Smith, 1931)</td>
<td>Giant catfish</td>
</tr>
</tbody>
</table>
Table 2. Morphologic similarities and differences between *Pangasius bocourti* and *Pangasianodon hypophthalmus* (modified according to Roberts & Vidthayanon, 1991).

<table>
<thead>
<tr>
<th>Species name</th>
<th><em>Pangasius bocourti</em></th>
<th><em>Pangasianodon hypophthalmus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Head shape</strong></td>
<td>Relatively narrow, slightly cuspidate/truncate or rounded</td>
<td>Moderately broad truncate, cuspidate in lateral view</td>
</tr>
<tr>
<td><strong>Mouth shape</strong></td>
<td>Sub-terminal; when the jaw is closed, the upper jaw is partially or largely exposed</td>
<td>Terminal; The tooth band in the upper jaw is completely covered by the lower jaw when the mouth is closed</td>
</tr>
<tr>
<td><strong>Palatal tooth bands</strong> (Reference)</td>
<td>Process a broader vomerine toothplate</td>
<td>Palatine plates joined to a single curved toothplate while vomerine ones are separated at the middle lines</td>
</tr>
<tr>
<td><strong>Gill rakers</strong></td>
<td>High counts of relatively shorter gill rakers between 36 to 46, arrange in descending/ascending size</td>
<td>High counts between 29 to 38; Characterized by alternate arrangements of small and big rakers</td>
</tr>
<tr>
<td><strong>Swim bladder</strong></td>
<td>Two-chambered swing bladder</td>
<td>Single swim bladder chamber extends to the posterior half of the anal fin basa</td>
</tr>
<tr>
<td><strong>Coloration</strong></td>
<td>Dull grey/black for dorsal parts or even green, bluish green, iridescent blue; white for ventral parts</td>
<td>A stripe in the middle of the anal fin under 30cm, and lose distinctive coloration for when grow over 40 or 50cm</td>
</tr>
<tr>
<td><strong>Vertebral counts</strong></td>
<td>Similar caudal vertebral counts between 25 to 27</td>
<td>Total counts are higher than 45, and more of the abdominal vertebrae (19 to 21) Between 40 to 45 of the total counts, and low counts of abdominal vertebrae (15 to 16)</td>
</tr>
</tbody>
</table>
Table 3. Types and numbers of fish samples collected in local restaurants.

<table>
<thead>
<tr>
<th>Sample types</th>
<th>Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A</strong> Catfish products</td>
<td>15</td>
</tr>
<tr>
<td><strong>Group B</strong> Products of fish species w/ higher prices</td>
<td></td>
</tr>
<tr>
<td>a. Grouper</td>
<td>18</td>
</tr>
<tr>
<td>b. Snapper</td>
<td>1</td>
</tr>
<tr>
<td>c. Sea bass</td>
<td>1</td>
</tr>
<tr>
<td>d. Sole</td>
<td>1</td>
</tr>
<tr>
<td><strong>Group C</strong> Products w/o notifying fish species</td>
<td>9</td>
</tr>
<tr>
<td><strong>In sum</strong></td>
<td>47</td>
</tr>
</tbody>
</table>
Table 4. General information of the restaurant samples including name, category, product type and its description, price and the result analyzed using the EZ™ Pangasius Kit.

<table>
<thead>
<tr>
<th>Name</th>
<th>Category</th>
<th>Type</th>
<th>Description</th>
<th>Price</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>r8</td>
<td>Catfish 1</td>
<td>Sandwich</td>
<td>Breaded and deep fried</td>
<td>$8.99</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r18</td>
<td>Catfish 2</td>
<td>Sandwich</td>
<td>Whole fillet, breaded and fried</td>
<td>$10.50</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r19</td>
<td>Catfish 3</td>
<td>Boneless file</td>
<td>Whole fillet, lightly breaded and fried</td>
<td>$9.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r20</td>
<td>Catfish 4</td>
<td>Fillet</td>
<td>Breaded and deep fried</td>
<td>$8.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r23</td>
<td>Catfish 5</td>
<td>Pontchartrain</td>
<td>Whole fillet, breaded and fried</td>
<td>$15.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r26</td>
<td>Catfish 6</td>
<td>Basket</td>
<td>Breaded and deep fried</td>
<td>$9.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r27</td>
<td>Catfish 7</td>
<td>Po' boy</td>
<td>Breaded and deep fried</td>
<td>$9.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r31</td>
<td>Catfish 8</td>
<td></td>
<td>Whole fillet, breaded and fried</td>
<td>$7.49</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r32</td>
<td>Catfish 9</td>
<td></td>
<td>Whole fillet, fried</td>
<td>$8.99</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r35</td>
<td>Catfish 10</td>
<td>basket</td>
<td>Breaded and deep fried</td>
<td>$9.99</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r41</td>
<td>Catfish 11</td>
<td></td>
<td>Breaded and deep fried</td>
<td>$5.38</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r42</td>
<td>Catfish 12</td>
<td></td>
<td>Breaded and deep fried</td>
<td>$11.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r45</td>
<td>Catfish 13</td>
<td>Sandwich</td>
<td>Breaded and deep fried</td>
<td>$9.75</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r47</td>
<td>Catfish 14</td>
<td>Platter</td>
<td>Breaded and deep fried</td>
<td>$8.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r49</td>
<td>Catfish 15</td>
<td></td>
<td>Breaded and deep fried</td>
<td>$9.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r2</td>
<td>Grouper 1</td>
<td>Sandwich</td>
<td>Grilled fillet</td>
<td>$10.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r3</td>
<td>Grouper 2</td>
<td>Sandwich</td>
<td>Grilled fillet</td>
<td>$11.00</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r6</td>
<td>Grouper 3</td>
<td>Sushi roll</td>
<td>Tempura, lightly breaded and fried</td>
<td>$6.00</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r7</td>
<td>Grouper 4</td>
<td>Finger for kids</td>
<td>Breaded and deep fried</td>
<td>$4.00</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r9</td>
<td>Grouper 5</td>
<td>Salad</td>
<td>Breaded and deep fried</td>
<td>$8.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r10</td>
<td>Grouper 6</td>
<td>Platter_dinner</td>
<td>Whole fillet, breaded and fried</td>
<td>$14.95</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r11</td>
<td>Grouper 7</td>
<td>Platter_lunch</td>
<td>Whole fillet, breaded and fried</td>
<td>$14.95</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r16</td>
<td>Grouper 8</td>
<td>Salad</td>
<td>Lighted breaded and cooked with spice</td>
<td>$10.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r21</td>
<td>Grouper 9</td>
<td>Sandwich</td>
<td>Whole fillet, grilled</td>
<td>$12</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r28</td>
<td>Grouper 10</td>
<td></td>
<td>Fried</td>
<td>$14.99</td>
<td>&quot;+&quot;</td>
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<tr>
<td>r29</td>
<td>Grouper 11</td>
<td></td>
<td>Grilled</td>
<td>$14.99</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r38</td>
<td>Grouper 12</td>
<td>Sandwich</td>
<td>Breaded and deep fried</td>
<td>$11.00</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r40</td>
<td>Grouper 13</td>
<td>Sandwich</td>
<td>Grilled</td>
<td>$11.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r43</td>
<td>Grouper 14</td>
<td>Tacos</td>
<td>Blackened</td>
<td>$16</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r44</td>
<td>Grouper 15</td>
<td>Sandwich</td>
<td>Grilled</td>
<td>$13.00</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r46</td>
<td>Grouper 16</td>
<td>Sandwich</td>
<td>Grilled, covered w/ sauces</td>
<td>$13.50</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r48</td>
<td>Grouper 17</td>
<td>Platter</td>
<td>Breaded and deep fried</td>
<td>$13.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r50</td>
<td>Grouper 18</td>
<td>Finger</td>
<td>Breaded and deep fried</td>
<td>$11.85</td>
<td>&quot;-&quot;</td>
</tr>
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</table>
Table 4 – continued.

<table>
<thead>
<tr>
<th>Name</th>
<th>Category</th>
<th>Type</th>
<th>Description</th>
<th>Price</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>r4</td>
<td>Snapper 1</td>
<td>Fingers</td>
<td>Breaded and deep fried</td>
<td>$12.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r5</td>
<td>Snapper 2</td>
<td>Po' boy</td>
<td>Breaded and deep fried</td>
<td>$9.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r17</td>
<td>Snapper 3</td>
<td>Asian cooking</td>
<td>Whole fish, skinned, breaded and fried</td>
<td>$23.00</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r14</td>
<td>Seabass</td>
<td>Asian cooking</td>
<td>Fried whole fillet, breaded</td>
<td>$10.00</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r24</td>
<td>Sole</td>
<td>Crusted</td>
<td></td>
<td>$14.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r1</td>
<td>w/o specifying species 1</td>
<td>Fish platter</td>
<td>Pan fried with spices, whole fillet</td>
<td>$8.25</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r12</td>
<td>w/o specifying species 2</td>
<td>Fish tacos</td>
<td>Small size whole fillet</td>
<td>$7.99</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r13</td>
<td>w/o specifying species 3</td>
<td>Mariachi</td>
<td>Whole fillet</td>
<td>$13.99</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r30</td>
<td>w/o specifying species 4</td>
<td></td>
<td>2 pieces, breaded and deep fried</td>
<td>$4.99</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r33</td>
<td>w/o specifying species 5</td>
<td>Tacos</td>
<td>Breaded and deep fried</td>
<td>$6.98</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r34</td>
<td>w/o specifying species 6</td>
<td>Sandwich</td>
<td>Breaded and deep fried</td>
<td>$3.19</td>
<td>&quot;-&quot;</td>
</tr>
<tr>
<td>r36</td>
<td>w/o specifying species 7</td>
<td>Wrap</td>
<td>Grilled, pieces</td>
<td>$6.95</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r37</td>
<td>w/o specifying species 8</td>
<td>Sandwich</td>
<td>Grilled, whole fillet</td>
<td>$6.95</td>
<td>&quot;+&quot;</td>
</tr>
<tr>
<td>r39</td>
<td>w/o specifying species 9</td>
<td>Burger</td>
<td>Breaded and deep fried</td>
<td>$6.25</td>
<td>&quot;-&quot;</td>
</tr>
</tbody>
</table>

"+" indicated Pangasius positive samples, and "-" represented Pangasius negative samples.
Figure 1. Principle of the EZ Pangasius™ Assay. The all-fish specific mAb F7B8 was used as the capture antibody, while the mAb T7E10, which could specially identify the Pangasius fish, was used as the detection antibody.

Figure 2. Different types of the restaurant fish samples and proportion of Pangasius positive samples.
Figure 3. Testing of restaurant samples by mAbs T7E10-based iELISA. The mAbs supernatant diluted 1:8 in antibody buffer were used in the assay. CF: catfish standard; T: tra/swai standard; B: basa standard; BG: black grouper standard. **a.** Representative catfish restaurant samples including four Pangasius (+) samples (r8, r32, r35 and r41) and six Pangasius (-) samples (r18, r19, r23, r27, r42 and r45); **b.** Representative grouper restaurant samples including four Pangasius (+) samples (r10, r11, r28 and r29) and six Pangasius (-) samples (r2, r7, r9, r21, r43, r46); **c.** Representative restaurant samples without specifying species and snapper/sea bass samples. The r1, r12, r13, r33, r36, r37 were Pangasius (+) samples while r34 and r39 were Pangasius (-) samples; **d.** Follow-ups of representative restaurant samples. The r8f, r32f, r35f and r41f belonged to the catfish group while r1f, r12f and r33f were samples without specifying species. n=2.
Figure 4. IEF protein banding pattern of restaurant “catfish” samples in polyacrylamide gel, pH 3.5-9.5. Anode was on top of the gel. STD: pI marker, rB: raw basa, cB: cooked basa, rS: raw swai, cS: cooked swai, rC: raw channel catfish, cC: cooked channel catfish, 1-4: restaurant Pangasius positive samples, 5-15: restaurant Pangasius negative samples.

Figure 5. IEF protein banding pattern of representative restaurant “grouper” samples in polyacrylamide gel, pH 3.5-9.5. Anode was on top of the gel. STD: pI marker, rB: raw basa, cB: cooked basa, rS: raw swai, cS: cooked swai, rBG: raw black grouper, cBG: cooked black grouper, rRG: raw red grouper, cRG: cooked red grouper, 1-4: restaurant Pangasius positive samples, 5-8: restaurant Pangasius negative sample.
Figure 6. SDS-PAGE profiles of the restaurant Pangasius (+) samples. The gel was run at 200V for 45mins. Each lane was loaded 8µg protein in 15µl extract. STD: Precision Plus Protein Kaleidoscope Standard; cB: cooked basa standard; cT: cooked tra/swai standard; cC: cooked catfish standard; rB: raw basa standard; rT: raw tra/swai standard; a. The r8, r32, r35 and r41 were Pangasius (+) samples in the catfish group while r10, r11, r28, r29 were Pangasius (+) samples in grouper category. The r20 was one Pangasius (-) sample; b. The r1, r12, r13, r33 and r36 were restaurant Pangasius (+) samples without specifying species.
Figure 7. Antigenic protein banding patterns of the restaurant samples using mAb T1G11. The mAb T1G11 supernatant which was concentrated to half of its original volume was used as the primary antibody, and goat anti-mouse IgG-AP conjugate diluted 1:3000 was used as secondary antibody. T: tra/swai standard; B: basa standard; r28, r29, r10, r11 were Pangasius (+) samples in grouper category, and r8, r32, r35 and r41 were Pangasius (+) samples in the catfish group.
Figure 8. Box plot for the price of Pangasius negative and positive samples. And it implied a symmetric and positively skewed distribution for Pangasius negative and positive respectively. Outliners indicated extreme sample prices of $23, $4.00 and $3.19.
REFERENCES


application to detect white fish species in food products and an interlaboratory study. *Journal of agricultural and food chemistry*, 53(9), 3348-3357.


BIOGRAPHICAL SKETCH

EDUCATION

2013.08–present  **Master student**, Food Sciences, Department of Nutrition, Food and Exercise Sciences, Florida State University, Tallahassee, FL,

2010.09–2012.07  **Master of Science in Molecular Genetics and Biotechnology**, Department of Biology, Lund University, Lund, Sweden

2005.09–2009.07  **Bachelor of Science in Biology**, Department of Biology, AnHui Normal University, Wuhu, China

RESEARCH EXPERIENCE

2013.08–present  Department of Nutrition, Food and Exercise Sciences, Florida State University, Florida, USA
  Project: Identification of commercial fish species using protein-based methods

2011.09–2012.07  Department of Plant Breeding and Biotechnology, Swedish University of Agricultural Science, Alnarp, Sweden
  Master Thesis Project: Genetic modification of Crambe abyssinica for better understanding of the biosynthesis pathways of erucic acid

Department of Biology, Lund University, Sweden


2011.04–2011.05  Mutagenesis project: Isolation of ToTK1 (Tomato Thymidine kinase 1) mutations as better candidates for suicide gene therapy
2010.11−2011.01  Mutagenesis project: Isolation of ftsZ mutants in Streptomyces coelicolor

TEACHING EXPERIENCE

2014.08‒2015.04  Graduate Teaching Assistant, Department of Nutrition, Food and Exercise Sciences, Florida State University
   Course: FOS 414C Food Science Laboratory (Fall 2014, Spring 2015)

AWARDS AND AFFILIATION

2014–Current  Student Member of Institute of Food Technologists (IFT)

Florida-China Linkage Institute  Tuition Exemption Award 2014

Florida State University  Jacqueline Boudier Clemens & Leslie E. Clemens Scholarship 2014

Florida State University  Betty M. Watts Memorial Fund 2014

Florida State University  The College of Human Sciences’s Research & Creativity Day Oral Presentation Third Place 2014

PUBLICATIONS AND BOOK


PRESENTATIONS


SERVICES

Bakery Judge in the North Florida Fair, 2013.