Doctoral Dissertation

Characterization and Utilization as Food of Freshwater Eels Collected from Tributaries along Lagonoy Gulf, Philippines

フィリピンラゴノイ湾で採取されたウナギの特徴づけおよびその利用学的検討

by

KATRINA LASALITA CANON

Graduate School of Integrated Arts and Science

Kuroshio Science Program

Kochi University

September 2021

Acknowledgments

The researcher would like to extend her deepest and sincerest gratitude and appreciation to all the people and organizations who helped and assisted her in the success of this study. Gratefully acknowledged are the support and selfless assistance of the following:

Dr. Satoshi Kubota, her supervisor, for his expertise, assistance, guidance, considerations, patience and understanding during the whole PhD course which enabled the researcher to come up with this paper.

The dissertation committee: Dr. Izumi Kinoshita, Dr. Yohei Nakamura, and Dr. Maki Teramoto for the comments, suggestions, constructive criticisms, and recommendations about her study.

Dr. Plutomeo M. Nieves, for his continued support, assistance, and encouragements throughout this study; his Eel Fishery Project Team: Prof. Antonino B. Mendoza, Dr. Raul G. Bradecina and the research assistants John Christopher Nolial, Niño C. Celestial, Ronie O. Mediario for the sample collection, patiently answering query related to the study.

The fishers and collectors for their assistance during the sampling and participation as respondents in the interview.

The Bicol University and Prof. Maria Asuncion V. Oronan, for their great help in recommending the researcher for a scholarship.

The Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, for the funds, being a Monbukagusho MEXT Scholarship (2018-2021) under the Program of Nurturing Talented People to Establish a Sustainable Society in the Kuroshio Region, which supported the conduct of the study and the researcher's everyday life in Japan; the Department of Science and Technology-Philippine Council for Agriculture, Aquatic, and Natural Resources Research and Development (DOST-PCAARRD), for the approval and grant of The Eel Fishery in Tributaries Along Lagonoy Gulf: Implications for Conservation and Management Project for which this study was supported in part; the Department of Agriculture-Bureau of Fisheries and Aquatic Resources (DA-BFAR) for the approval of pertinent permits for the conduct of the study and transport of the samples from Philippines to Japan; the Local Government Units of Malinao, Albay, Lagonoy, Camarines Sur, and Bato Catanduanes for their support and cooperation during the sampling; and the Research Instrument and

Radioisotope Research Division of Biological Research, Science Research Center, Kochi University for DNA sequencing analysis results.

The laboratory members: Joshua, Sam, Teresa, and Dan for their assistance in sample preparation, data analysis, proofreading of the paper and presentation, and their moral support during the tough times throughout the three years as a student. Alvin, Jonel, Janice, Anabelle, Liezel, Donna, and Kristian for the encouragement and pieces of advice.

Dr. Yoshinori Morooka, for believing in the researcher's capability, his unwavering support, and encouragements; Dr. Akira Tominaga and Dr. Takahiro Taguchi for their assistance and encouragement.

The researcher's family and friends, especially Mom and Mommy that served as her inspiration. In as much, great thanks for their unconditional love, valuable support, emotionally and financially, for their continuous prayers that enlightened her mind to complete the study.

To all those who were not mentioned, however, in one way or another has imparted help in the realization of this paper; the researcher would like to extend her sincerest appreciation.

Above all, our Creator, Almighty God, for the wisdom, courage, confidence, strength, good health, blessings, and guidance He has given that contributed to the realization of this study.

To everyone mentioned, this paper is dearly dedicated.

カトリーナ　ラサリタ　キャノン

K. L. Canon

Table of Contents

List of Tables

List of Figures

List of Main Papers

Main papers used in creating the dissertation

Peer-reviewed papers

1. Canon, Katrina L., Nieves, Plutomeo M., Mendoza Jr., Antonino B., Nolial, John Christopher C., Celestial, Niño C., Mediario, Ronie O., Bradecina, Raul G., Avila, Teresa N., and Kubota, Satoshi. (2021) Occurrence of *Anguilla luzonensis* in the tributaries along the Lagonoy Gulf, Philippines, Journal of Fisheries Science, 3(1): 8-16

Additional papers

1. Taguchi, Takahiro, Tagami, Erika, Mezaki, Takuma, Vacarizas, Joshua Magnaye, Canon, Katrina Lasalita, Avila, Teresa Narvaez, Bataan, Dan Anthony Uy, Tominaga, Akira, and Kubota, Satoshi. (2020). Karyotypic mosaicism and molecular cytogenetic markers in the scleractinian coral *Acropora pruinosa* Brook, 1982 (Hexacorallia, Anthozoa, Cnidaria). Coral Reefs, 39:1415–1425

2. Nieves, Plutomeo M., Mendoza, Antonino B., Bradecina, Raul G., Nolial, John Christopher, Celestial, Niño C., Kubota, Satoshi., and Canon, Katrina L. (2021) The Eel Fishery in Tributaries Along Lagonoy Gulf: Implications for Management and Conservation. BU R&D Journal: Bicol University, 24(1), 67-81

3. Avila, Teresa N., Bigueja, Myrna C., Bradecina, Raul G., Mendoza, Jr., Antonino B., Canon, Katrina L., and Kubota, Satoshi. (In press). Proximate composition and changes in muscle proteins of dried salted abo (*Otolithes ruber*). Food Research

List of Conference Presentations

1. Katrina L. Canon, Plutomeo M. Nieves, Antonino B. Mendoza Jr., John Christopher C. Nolial, Niño C. Celestial, Ronnie O. Mediario, Raul G. Bradecina, Teresa N. Avila, and Satoshi Kubota, Species Composition of Freshwater Eels collected from Tributaries Along Lagonoy Gulf, Philippines using PCR-RFLP Analysis. 13th International Kuroshio Science Symposium / Cagayan, Philippines, November 19-21, 2019

2. Katrina L. Canon, Plutomeo M. Nieves, Antonino B. Mendoza Jr., John Christopher C. Nolial, Niño C. Celestial, Ronnie O. Mediario, Raul G. Bradecina, Teresa N. Avila, and Satoshi Kubota, Species Composition of Anguillids Along Lagonoy Gulf, Philippines, 2020 Japanese Society of Fisheries Science (JSFS) Spring Meeting/ Tokyo, Japan, March 26-30, 2020

Chapter 1 General Introduction

1.1 Market and trade

Anguilla japonica, *A. anguilla*, and *A. rostrata* are freshwater eel species of commercial importance worldwide (Ringuet et al., 2002). Among these species, there is an increasing demand for *A. japonica* in East Asian countries such as China and Japan (Food and Agriculture Organization (FAO), 2009), with annual consumptions that reached 150,000-160,000 tonnes between 2000 and 2013 (Shiraishi & Crook, 2015). As a result, aquaculture production increased from 69,876 tonnes in 1980 to 263,403 tonnes in 2018 (FAO, 2020). However, *A. japonica* capture production had been on the decline, from 2,535 tonnes in the 1980s to 118 tonnes in 2018 (FAO 2020), due to overfishing and environmental changes (Aoyama et al., 2015; Japan Wildlife Conservation Society (JWCS), 2017; Ringuet et al., 2002). The gap between aquaculture and capture production had been continuously fulfilled by the import of *Anguilla* spp. (Crook, 2014). In 2012 and 2013, approximately 30% of all East Asian live elvers and glass eel imports were sourced from the Philippines (Crook, 2014).

In the Philippines, the annual catch has increased by 100 or 1,000 tonnes per year from 2000 to 2019 (FAO, 2020; PSA, 2020). Also, aquaculture export has increased from 98,000 to 25,000,000 USD between 2000 and 2017 (UN Comtrade, 2020). Glass eels and elvers comprise the greater part of these exported resources (Crook, 2014; Cuvin-Aralar et al., 2019; Southeast Asian Fisheries Development Center (SEAFDEC), 2019). Within the Philippines, Cagayan River have been considered the major fishing grounds for glass eels and elvers, mainly *A. marmorata* and *A. bicolor pacifica* (Crook, 2014). The collection of these species has also expanded to Mindanao (Crook, 2014). *Anguilla marmorata* was found to be abundant in both these areas (Aoyama et al., 2015; Shirotori

et al., 2016). Although *A. marmorata* is the most-abundant species, a higher percentage of *A. bicolor pacifica* (87 %) is being exported (Asis et al., 2014).

1. 2 Freshwater eel as food and its composition

Freshwater eels have long been consumed as food. Some countries have eels as part of their traditional cuisine. Eels were hot smoked and traditionally consumed in Demark, Ireland, and New Zealand (Jellyman, 2014; McCarthy, 2014; Rindom et al., 2014). Jellied eels were also a traditional dish in the United Kingdom, wherein chopped eels are just boiled in stock and set (Righton & Roberts, 2014). Further, juveniles eels tossed in olive oil with garlic and pepper are still served as an appetizer in Spain during special occasions (Randolph, 2018). In Asian countries, grilling of eel (*kabayaki*) is common and the most popular dish of freshwater eel, which is customarily consumed in Japan, China, Taiwan, and Korea (Dou, 2014; Kuroki et al., 2014; Lee, 2014; Tze 2014).

Studies on analysis of tastes and histology have been carried out for some freshwater eels. Yang and Lee (1980) determined the taste components of wild *A. japonica*, wherein it included free amino acids (FAA), nucleotides and the related compounds, organic bases, sugars, organic acids, and minerals. The chemical analysis and organoleptic test undergone a triangle difference test and showed that glycine, serine, glutamic acid, IMP, lysine, alanine, isoleucine, aspartic acid, creatine, and ions of Na, K, Cl, and phosphate were the major components contributing to taste of the eel. Changes in the muscles were also observed when eels are dried or frozen. Song et al. (1982) showed that during the early stage of drying, the connective tissue of the muscle was dehydrated. During the later stage of drying, dehydration in the muscle fiber, movement of fat, and structural changes of the myocommata were observed. Song and Lee, (1982) showed that

storage at very low temperatures showed ice crystals outside (-20 °C) and inside (-40 °C) the muscle cells. Further, thawing did not recover the structure of the muscular tissue. Ersoy et al. (2008) also compared the effects of different thawing methods on the quality of frozen eels, showing that water thawing the most suitable method. Moreover, Özogul et al. (2005) has assessed the freshness of eel fillets stored in a refrigerator with ice and without ice for 19 days. The total viable count determined the shelf-life of the eel in ice as 13-14 days and 6-7 days for those stored without ice. The chemical analysis, i.e., total volatile base- nitrogen, peroxide value, showed that the flesh started to spoil after five days of storage. However, the oxidation of lipid was only evident after the 8th day of storage. Among the freshwater eels, *A. marmorata* has been considered an underutilized species (Cheng et al., 2020). With the continuously growing population, the need to explore the potential for underutilized species is particularly pressing. Focusing on the underutilized species and effective processing methods may lead to new products and create a new market, thereby, studies on chemical composition and other aspects of food development have been carried out.

Nutritional composition analyses were also carried out for the species of freshwater eels. For the cultured eels, the proximate composition analysis showed that *A. marmorata* has higher moisture content compared with *A. japonica*, *A. bicolor pacifica*, *A. rostrata*, and *A. Anguilla* (Ahn et al., 2015; Luo et al., 2015; Zhiyong, 1998) but lower than *Anguilla bicolor bicolor* (Nafsiyah et al., 2018). Further, the fat content of *A. marmorata* was found to be lower compared with *A. japonica*, *A. bicolor pacifica*, *A. rostrata*, and *A. anguilla* (Ahn et al., 2015; Luo et al., 2015; Zhiyong, 1998) but higher than *A. bicolor bicolor* (Nafsiyah et al., 2018). In terms of the protein content, higher values were observed in *A. marmorata* compared with *A. bicolor pacifica*, *A. anguilla*, *A. japonica*, and *A. rostrata* (Ahn et al., 2015; Luo et al., 2015). However, ash content was

almost similar among all the species mentioned earlier (Ahn et al., 2015; Luo et al., 2015; Nafsiyah et al., 2018). Zhiyong (1998) has compared the amino acids (AA) content between *A. marmorata* and *A. japonica*, wherein *A japonica* has higher AA content, yet all the essential amino acids were also found in *A. marmorata*. Ahn et al. (2015) showed that the amino acid (AA) contents of *A. marmorata*, *A. japonica*, *A. bicolor pacifica*, and *A. rostrata* were almost comparable. Luo et al. (2015) compared the AA of 5 species of cultured freshwater eels — *A. marmorata*, *A. bicolor pacifica*, *A. japonica*, *A. rostrata*, and *A. anguilla*. Comparable values for isoleucine, methionine, valine, serine, and tyrosine were observed among the five species, and other AA has varying contents. On the other hand, AA of wild-caught *A. marmorata* has higher histidine, lysine, methionine, arginine, and tyrosine compared with the cultured ones, while the other AA was found to be almost similar between these two (Ahn et al., 2015; Jamaluddin et al., 2019; Luo et al., 2015; Nafsiyah et al., 2018; Zhiyong, 1998). In terms of the fatty acids, ratios of the saturated and polyunsaturated fatty acids of *A. japonica*, *A. bicolor pacifica*, and *A. marmorata* were said not to show any significant difference (Luo et al., 2015). However, EPA and DHA were higher in the tropical eels than in temperate eels (Ahn et al., 2015). The monounsaturated and polyunsaturated fatty acids were higher in *A. marmorata* than *A. bicolor bicolor* (Nafsiyah et al., 2018). In as much, vitamin A of *A. marmorata* was higher than *A. bicolor pacifica*, *A. japonica*, *A. rostrata* (Ahn et al., 2015), and *A. bicolor bicolor* (Nafsiyah et al., 2018). Mineral contents of the freshwater eel muscle were also determined. Zhiyong (1998) showed that essential metals (Ca, P, Na, Mg, K, Zn, Al, and Mn) are higher in *A. marmorata* than *A. anguilla* and *A. japonica*. Luo et al., (2015) also showed that the Mg, K, and Zn level in *A. marmorata* is higher than *A. anguilla*, *A. japonica*, and *A. rostrata*. Also, *the A. marmorata* P level is next to the highest content of *A. japonica*. On the other hand, wild-caught *A. marmorata* has higher Mg but lower K

and Zn than the aquacultured one (Jamaluddin et al., 2019; Luo et al., 2015). Although heavy metals were also found to accumulate in the flesh of wild-caught *A. marmorata*, levels did not pose a risk for human consumption (Le et al., 2009). Further, the accumulation of trace metals in the flesh of *A. marmorata* and *A. bicolor pacifica* was higher in silver eels than in yellow eels (Le et al., 2012).

Separately, Ahn et al., (2015) has conducted a preference test wherein expert panels underwent a blind taste test of the flesh of *A. marmorata*, *A. japonica*, *A. bicolor pacifica*, and *A. rostrata* where it showed that *A. marmorata* ranked 2nd to *A. japonica*. The chewy taste of the flesh was also evaluated, wherein *A. marmorata* has obtained a seemingly advanced evaluation compared with *A. japonica*. Sato et al. (1986) have emphasized that the collagen content of fish muscle affects the texture of its meat. Fresh eel meat texture, i.e., *A. japonica*, showed high collagen content and tough texture based on the sensory evaluation. However, the cooked meat texture was more tender, succulent, and elastic due to the high collagen content in the muscle. *Anguilla japonica* (01.41-1.99% wet tissue; 8.8-12.4% of crude protein) and *Conger myriaster* (2.19% wet tissue; 11% of crude protein) were reported to have the highest total collagen in the ordinary muscle in the dorsal part of the trunk among fishes belonging to 24 species (Sato, Yoshinaka, Sato, & Shimizu, 1986). *Anguilla marmorata* and *A. bicolor pacifica* which is of the same genus with *A. japonica* may also have high collagen content.

Collagen, a fibrous protein, is abundant and the main component of the extracellular matrix (Alberts et al. 2002, 2015). Boiling denatures triple helix structure of collagen thereby producing gelatin (Tornberg, 2005; Belitz et al., 2009; Alfaro et al., 2015). Gelatin was reported to be a taste enhancer (Kuroda et al. 1997; Boran & Regenstein, 2010; Qi et al., 2020). *Paksiw* is one of the famous cuisine in the Philippines, prepared by boiling fish with water, vinegar and spices (Fernandez, 1988). *Anguilla*

marmorata and *A. bicolor pacifica* cooked in the same way as *paksiw* will have gelatin in its soup that can enhance its taste.

Cheng et al. (2020) has utilized *A. marmorata* to make eel protein hydrolysates (EPH) by enzymatic hydrolysis of alcalase, bromelain, and papain. Essential AA (His, Thr, Val, Ile, Leu) of the alcalase hydrolyzed EPH showed comparable World Health Organization's (WHO) recommended daily intake of EAAs needed to meet the protein requirement for an adult.

The studies have shown that the moisture, ash, and amino acid content of *A. marmorata* is comparable with *A. bicolor pacifica*. The fat content of *A. marmorata* was lower, while the protein and vitamin A content was higher than *A. bicolor pacifica*. On the other hand, no significant differences were observed among the saturated and unsaturated fatty acids of *A. marmorata* and *A. bicolor pacifica*. Moreover, the preference test revealed that *A. marmorata* is of a higher rank than *A. bicolor pacifica*. These data indicated that *A. marmorata* might have the potential for food utilization. The possibility of developing this species into food products can be explored to fully maximize the resources available and abundant in an area. In turn, this may create new markets and avenues for the fishers to improve their livelihood.

1.3 Species identification

Species are traditionally identified based on the morphological characters of freshwater eels (Castle & Williamson, 1974; Tabeta et al., 1976; Tabeta et al., 1976; Watanabe et al., 2004).

Ege (1939, as cited in Aoyama, 2009) has classified the 18 species of freshwater eels based on the morphological characters. The comparison of the color and markings divided the species into two groups as species with variegated markings and without

variegated markings. These were then further divided into three groups combining the character for dorsal, anal, and total length relationships with shortfin species without variegated markings, longfin without variegated markings, and longfin with variegated markings. The longfin species with variegated markings were further divided into two groups based on the dentition as an undivided maxillary band and the toothless longitudinal groove in the maxillary band. Thereby classifying the 18 species into four groups based on the following: A) *A. celebesensis*, *A. interioris*, and *A. megastoma* as variegated species with broad, undivided maxillary, and mandibular bands of teeth; B) *A. nebulosa nebulosa*, *A. nebulosa labiata*, *A. marmorata*, *A. reinhardtii* and *A. ancestralis* (later synonymized as *A. celebesensis* by Castle and Williamson, 1974) variegated species with a toothless longitudinal groove in the maxillary and mandibular bands of teeth; C) *A. borneensis*, *A. japonica*, *A. dieffenbachii*, *A. Anguilla*, *A. rostrata*, and *A. mossambica* as longfin species without variegated marking; and D) with *A. bicolor bicolor*, *A. bicolor pacifica*, *A. obscura, A. australis australis*, and *A. australis schmidtii* species group with short fin and no variegated markings.

Watanabe et al. (2004) reexamined this classification using the same characters by Ege (1939) and two modified characters. The qualitative characters for the dentition (maxillary band and teeth) were converted to quantitative characters as proportions of the width of the midpart to the length of the maxillary band and number of the teeth of the midpart of the maxillary band. Base on the validation, the four groups were characterized as A) Variegated skin with broad maxillary bands of teeth; B) Variegated skin with narrow maxillary bands of teeth; C) Non-variegated skin with long dorsal fin, and D) Non-variegated skin with a short dorsal fin. Groups C and D were exactly grouped the same as Ege (1939), while groups A and B were partly different since the modified characters were used, making the groupings more precise compared with Ege's (1939)

(Watanabe, 2003). For identification of each of the species, geographic distribution was concluded to be important in Ege's (1939) study. However, specimens from one locality wherein species group's distribution overlaps are difficult to identify using their morphology (Aoyama, 2009; Watanabe, 2003; Watanabe et al., 2004).

Another study has classified the 18 species of freshwater eels into seven groups. (Tesch & Greenwood, 1977) referred to Ege's (1939) classification and identification of the genus *Anguilla* using morphological characters. The four groups were further divided into seven groups taking into account the tooth evenness or unevenness and the narrowing before or after the middle plate of the tooth. It has been specified that the use of coloration and markings of the adults in the yellow eel stage easily separate the 18 species into two groups. The same qualitative and quantitative characters of each species were combined and further classified the species into seven groups, with one species being identified. However, these color patterns, i.e., marbled patterning disappears during the adult at silver eel stage. Even though this character is not evident at the silver eel stage, the species were still separated into seven groups and identified one species as in the former. Hence, not all morphological characters are useful in the distinction of each of the species. However, *A. japonica*, *A. marmorata*, *A. bicolor pacifica*, and *A. celebesensis* belonging to different groups and distributions not overlapping in the Philippines can be discriminated using these morphological characters.

For the glass eels and elvers, Tabeta et al. (1976) concluded that the sectional counts of the vertebrae were useful for the identification of 9 freshwater eels. The sectional vertebrae were counted after the specimens were treated with alizarin and x-ray photographed, dividing the nine species into two groups as species with ano-dorsal vertebrae below two and more than six. The group *A. bicolor pacifica*, *A. bicolor bicolor*, and *A. australis schmidti* are species with ano-dorsal vertebrae less than 2. Species with

ano-dorsal vertebrae more than six can be distinguished further using the total vertebrae count of more than 112 and less than 111. *Anguilla japonica* and *A. Anguilla* with more than 112 total vertebrae can be discriminated by the ano-dorsal vertebrae of 7-10 and 10-14, respectively. At the same time, species with less than 111 total vertebrae were further distinguished by the pre-dorsal vertebrae counts of 22-27 and 26-30. *Anguilla celebesensis* and *A. borneensis* have 22-27 pre-dorsal vertebrae, while *A. marmorata* and *A. rostrata* with 26-30. *Anguilla marmorata* and *A. rostrata* were further separated using the ano-dorsal vertebrae counts of 13-17 and 6-12, respectively. Importantly, the pre-dorsal and ano-dorsal vertebrae counts of the species with total vertebrae of less than 111 and more than 112 still overlap to some extent making the distinction of some species difficult. In addition to the sectional counts of vertebrae, the caudal cutaneous pigmentation facilitated the identification of the species by observation under a stereomicroscope (Tabeta et al., 1976). These include *A. japonica* lacking caudal cutaneous pigmentation and *A. bicolor pacifica* with small spots that appear first on the caudal fin and developed anteriorly during growth. On the other hand, *A. marmorata* and *A. celebesensis* with the same caudal cutaneous pigmentation can be separated only by using the numbers of ano-dorsal vertebrae. Therefore, *A. japonica* and *A. bicolor pacifica* can be identified using the combination of sectional counts of vertebrae and caudal pigmentation. However, *A. marmorata* and *A. celebesensis* can be identified by the ano-dorsal vertebrae count. Hence, the use of the sectional counts of the vertebrae can identify the species separately than the use of the caudal pigmentation. Shinoda et al. (2015) has initially classified glass eels by fin difference and caudal pigmentation, wherein a group of longfin specimens with caudal pigmentation were found. *A. luzonensis* was identified genetically from this group.

On the other hand, leptocephali of the genus *Anguilla* were identified based on the measurements of myomeres, last vertical blood vessel, and length measurements—total, preanal and pre-dorsal lengths (Jespersen, 1942; Miller &Tsukamoto, 2004). Jespersen (1942) used the number of myomere from the start of the dorsal fin and the end of the gut to measure the ano-dorsal distance. The ano-dorsal distance divided the group of ten species into two as shortfin and longfin species with less than 5 for the former and more than 5 for the latter. *Anguilla bicolor* and *A. australis* for shortfin species and *A. interioris*, *A. megastoma*, *A. celebesensis*, *A. marmorata*, *A. reinhardti*, *A. dieffenbachii*, and *A. japonica*. Then each of the species can be identified using the ano-dorsal distance except for *A. dieffenbachii* and *A. megastoma*. The range of the number of vertebrae among the longfin species can distinguish *A. japonica* from the other species except for *A. dieffenbachii*, wherein the range of measurements overlaps to some extent. *Anguilla dieffenbachii* can be separated from other species except for *A. japonica*, *A. megastoma*, and *A. reinhardti* due to some overlap in the range of measurements. *Anguilla interioris* and *A. megastoma* can be distinguished from each other using the total myomere counts. However, their measurements overlap with the other species, i.e., *A. interioris, A. borneensis, A. celebesensis, A. marmorata*, and *A. reinhardti*. Further, leptocephali which are not morphologically developed, length <20mm, cannot be identified using these morphological characters. Therefore, *A. bicolor pacifica*, the shortfin species, and *A. japonica*, *A. marmorata*, and *A. celebsensis* long fin species can be identified. Leptocephali of *A. luzonensis* were also found by (Kuroki et al., 2012) near the West Mariana Ridge and not far offshore to the east of Luzon, Philippines. Morphology showed that the specimens were longfin and have an overlapping range of total myomeres with *A. marmorata* and *A. celebesensis*. Distinctively, the shape of leptocephali is slightly more

slender than the other long-finned species. DNA sequencing identified this specimen as *A. luzonensis*.

Several molecular studies using the mitochondrial DNA of some species were carried out to analyze evolutionary relationships and species identification of adults, early stages, and indistinguishable specimens of the genus *Anguilla* (Aoyama et al., 1996; Aoyama & Tsukamoto, 1997; Tagliavini et al., 1996). In fact, the new species *Anguilla luzonensis* was identified to be genetically distinct from other species using DNA sequencing (Watanabe et al., 2009). These DNA sequences were used to develop fast and reliable molecular techniques for species identification. Aoyama et al. (2000) developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method using six restriction enzymes wherein 14 species were distinguishable. The PCR-RFLP methods were able to identify *A. rostrata*, *A. reinhardti*, *A. obscura*, *A. nebulosa nebulosa*, *A. mossambica*, *A. megastoma*, *A. marmorata, A. japonica*, *A. interioris*, *A. dieffenbachi*, *A. celebesensis*, *A. bicolor*, *A. australis*, *A. Anguilla,* and *A. borneensis*. Since this method has used six restriction enzymes to identify 14 species, the use of one restriction enzyme combined with morphology or locality and Ege's (1939) key may be enough to save more cost and time in the procedure. Another study has developed two molecular methods for species identification. The PCR-RFLP method using two restriction enzymes was applied for four species and distinguished *A. japonica*, *A. Anguilla*, *A. rostrata*, and *A. marmorata*. On the other hand, allele-specific PCR was used for two species and distinguished both *A. japonica* and *A. marmorata*. Although the allele-specific PCR was considered the cheapest and easiest to handle, an appropriate specific primer for each of the 14 species must be designed first before use and sometimes amplified separately. However, PCR-RFLP, which is also a cheap and fast method, usually uses one primer for a target gene and may be amplified and digested using the

same profiles. Nevertheless, *A. japonica*, *A. celebesensis*, and *A. marmorata* can be identified by molecular methods, i.e., PCR-RFLP.

Studies comparing morphological and molecular identification were also carried out. Watanabe et al. (2005) used six restriction enzymes from Aoyama et al. (2000) plus four other enzymes for PCR-RFLP to compare molecular and morphological identification of the genus *Anguilla*. The four groups based on morphology were further divided into 14 taxa based on the molecular analysis. Further, one in the 14 taxa was divided into 2 using the number of vertebrae making a total of 15 taxa which was then matched with 15 species. It was concluded that the molecular characters are more suitable to be compared with morphology for precise species identification. In addition, it was emphasized that establishing molecular techniques should use a large number of samples to observe and clarify genetic variations among and within species to identify each of the specimens at the species level, which was not shown in the studies as mentioned earlier. Sugeha et al. (2008) initially classified specimens only as shortfin or longfin species based on morphology for the freshwater eels at the glass eel stage. However, *A. bicolor bicolor*, *A. nebulosa nebulosa*, *A. bicolor pacifica*, *A. interioris*, *A. borneensis*, *A. celebesensis*, *A. marmorata*, *A. obscura*, and *A. megastoma* were identified by the PCR-RFLP method. Further, it was confirmed that morphology is not enough by comparing species identification with PCR-RFLP analysis. Sugeha & Arai (2010) have also compared the morphology, and molecular identification of *A. marmorata* collected from different locations. The morphology and PCR-RFLP patterns were also confirmed to be different among the glass eel of *A. marmorata* from two sites, indicating the possibility of different populations of this species. As such, we can only assume that PCR-RFLP methods needed refinement, taking into account the species found in a particular location, i.e., *A. bicolor*

pacifica, *A. japonica*, *A. marmorata*, *A. celebesensis*, and including the new species *A. luzonensis*.

Watanabe et al. (2009) confirmed the existence of a new species, *A. luzonensis*, genetically. *Anguilla luzonensis* was found to belong in group I species characterized as longfin species with variegated markings on the skin and undivided maxillary band. Morphological characters of adult specimens were further investigated by comparison with *A. celebesensis* and *A. ambonensis*, a junior synonym of *A. celebesensis*. Although *A. luzonensis* and *A. celebesensis* have similar morphology, statistically significant morphological differences were still observed. Teng et al. (2009) also genetically confirmed *A. huangi* sp. nov. from cultured eels. Morphology of the adult eel was characterized and found to be similar to *A. celebesensis*. *A. luzonensis* and *A. celebesensis* cannot be identified by morphology alone. Yet the DNA sequence is distinct from all the 18 species and subspecies of freshwater eels. Watanabe et al. (2013) have compared *A. luzonensis* and *A. huangi* based on morphology and molecular characters. *Anguilla luzonensis* specimen, which was from the wild, and *A. huangi*, reared in an aquaculture system, differ significantly in predorsal-fin, preanal, trunk, and gape measurements lengths. However, the abdominal and total number of vertebrae did not significantly differ, wherein it was stated that these two characters are not affected by the aquaculture process. Further, these two species were concluded to be genetically the same since there are only two different sites and a 0.003 genetic distance. Moreover, Minegishi et al. (2009) had evaluated the genetic divergence of the 19 species, which included the new *A. luzonensis*, wherein it showed that this species is distinct from the other 18 species.

Several studies have combined morphology and molecular techniques to identify freshwater eels at the glass eel stage. Han et al. (2012) has easily identified *A. bicolor pacifica* and *A. japonica* based on the fin-type (short) and the absence of the caudal

pigmentation. While *A. celebesensis*, *A. luzonensis*, *A. marmorata*, and *A. interioris* were identified through species-specific PCR and only those specimens that failed to show PCR bands were identified directly by DNA sequencing. Leander et al. (2012) initially observed the morphological characters for fin type and caudal pigmentation, which identified *A. bicolor pacifica* (shortfin) and *A. japonica* (longfin without caudal pigmentation). *Anguilla marmorata*, longfin with caudal pigmentation, was identified using the ano-dorsal and total length relationship expressed in percentage (>13%). Further, *A. marmorata*, *A. celebesensis*, and *A. luzonensis* were identified by DNA sequence analysis. Yoshinaga et al. (2014) also carried out a similar method wherein *A. bicolor pacifica* (short-finned), and *A. japonica* (pigment absent long-finned species) were identified. All the *A. japonica* specimens underwent DNA sequencing to verify the morphological identification. Also, *A. marmorata*, *A. celebesensis*, and *A. luzonensis* were identified from the subsamples of the longfin species that were DNA sequenced. Aoyama et al. (2015) were also able to identify *A. bicolor pacifica* and *A. japonica* morphologically by fin type and caudal pigmentation. Subsamples of these morphologically identified specimens were also confirmed genetically. In the initial sampling period, all the longfin specimens have undergone species-specific PCR adapted from (Han et al., 2012). Only those specimens having no bands were sequenced. For another period, random samples of the longfin specimens were sequenced for identification. *Anguilla marmorata*, *A. celebesensis*, and *A. luzonensis* were the species that were identified using molecular analysis. All the longfin specimens were *A. marmorata*, *A. celebesensis*, and *A. luzonensis*, as identified by DNA sequencing. Shirotori et al. (2016) has initially used caudal pigmentation, separating the specimens in three groups with pigmentation until the tail-tip, pigmentation not reaching the tail-tip, and pigment-absent species. The absent pigment species were initially assumed as *A.*

bicolor pacifica and *A. japonica*. Using the fin length, the specimens with pigment reaching the tail-tip were divided into two wherein *A. bicolor pacifica* was identified as having a short fin. Also, unknown specimens were found to have longfin and pigmentation similar to *A. bicolor pacifica*. All pigment-absent specimens, unknown specimens, and subsamples of longfin with caudal pigmentation not reaching tail-tip were subsequently analyzed by DNA sequencing for confirmation and identification. *Anguilla japonica* was the pigment-absent specimens; *A. interioris* and *A. borneensis* were the longfins with pigmentation until tail-tip; and *A. celebesensis*, *A. marmorata*, and *A. luzonensis* were the longfin with caudal pigmentation not reaching the tail-tip as identified by the direct sequencing method. Also, Han et al. (2016) initially identified the glass eels by fin difference and caudal pigmentation. *Anguilla bicolor pacifica* (shortfin) and *A. japonica* (longfin without pigmentation) can be identified morphologically without further molecular analysis. The same procedure in Han et al. (2012) was carried out for the specimens with fin difference below 13% (except for *A. bicolor pacifica*). *Anguilla marmorata*, *A. luzonensis*, and *A. celebesensis* were identified on species level by molecular analysis. In general, *A. bicolor pacifica* and *A. japonica* may be identified by observing their morphology. However, *A. celebesensis*, *A. marmorata*, and *A. luzonensis*, which cannot be distinguished using morphology, were identified by molecular analysis. Nevertheless, specimens that were already identified by morphology and those with unclear identification were confirmed using molecular analysis for precise species identification.

1.4 Spawning and distribution

Some species of freshwater eels inhabit the tropical regions wherein it is distributed in the temperate, tropical and subtropical areas (Aoyama et al., 2015; Han et

al., 2012; Leander et al., 2012; Shirotori et al., 2016; Sugeha et al., 2008; Watanabe et al., 2009). A series of an expedition by Japanese vessel was conducted from 1991 to 2009 at different years, principally designed to collect leptocephali of *A. japonica*. Various sizes of leptocephali were collected, and *Anguilla* leptocephali were immediately sorted out for subsequent analysis and preservation. *A. japonica* spawning site was first discovered in 1991 wherein 911 leptocephali ranging from 7.9 mm to 34.2 mm were collected within the margins of North Equatorial Current (Tsukamoto, 1992). Leptocephali was collected from 50-100 m depth and showed abundance at a depth of 75 m. The estimated spawning location of this species corresponds to a salinity front, separating the less saline water mass of the NEC from the typically high salinity of the tropical water (Tsukamoto, 1992). Hence, it would mean that the precise location of spawning may change with the location of the salinity front. More leptocephali (n= 1000) of this species were also collected in 1994 in a similar region. In 2005 to 2007, newly hatched preleptocephali and preleptocephali stages of *A. japonica* were still collected in the NEC region (Kuroki et al., 2006), suggesting that the spawning area of this species is indeed in the NEC region. In addition, the seamounts located in the westward flow of the North Equatorial Current are hypothesized to provide cues for migrating silver eels and serve as possible aggregation sites for spawning. It was further stated that *A. japonica* does not spawn continuously during the long spawning season from April to November but is synchronized to spawn periodically once a month during the new moon (Tsukamoto, 1992).

Also, during this expedition, leptocephali of other species were collected. *Anguilla marmorata* leptocephali with lengths ranging from 9-20 mm were consistently collected in the western north pacific within the NEC region during three consecutive cruises in 1991, 1994, and 1995 (Miller et al., 2002). Also, *A. marmorata* newly hatched preleptocephali and preleptocephali stages were collected in overlapping stations with *A.*

japonica during the surveys in 1998, 2004-2007 in the NEC region, indicating that these species have sympatric spawning sites and spawn in close proximity during summer (Kuroki et al., 2009). Further, *A. marmorata* leptocephali with sizes 9.6- 56.7 mm were collected in abundance in the NEC region to the west of the Mariana Islands (Kuroki et al., 2006). *Anguilla marmorata* has a year-round spawning (Miller et al., 2002). In 2005, 2006, and 2009, 5 leptocephali of *A. luzonensis* with sizes ranging from 4.3- 56.7 mm were collected in the west of Mariana ridge within the NEC region and off the coast near the east of Luzon Philippines (Kuroki et al., 2012). Therefore, it was believed that the *A. luzonensis* spawning site is located near the spawning grounds of *A. marmorata* and *A. japonica* and experience the same oceanographic features with these two latter species. However, it was concluded that further study on the early life stages of this new species is still necessary due to limited available information. Other than these species, large *A. bicolor pacifica* leptocephali size grouped as <35 mm and >45mm, were collected near NEC, North Equatorial Counter Current (NECC), and the South Equatorial Current (SEC) (Arai et al., 2001; Kuroki et al., 2006). Leptocephali with sizes >45 mm were collected from the SEC region. On the other hand, smaller leptocephali with sizes <35 mm were found in the NECC, wherein the current structure is variable; therefore difficult to speculate the exact spawning area (Arai et al., 2001).

Cruises in the southern part of the Western Pacific, Celebes Sea, Sulu Sea, and Tomini bay were also carried out of Japanese (2000) and Indonesian (1991-2002) vessels using identical trawls. Large leptocephali of *A. marmorata* (31.3-50.7 mm) were also collected in the area. Leptocephali of *A. celebesensis* (13.0-47.8 mm), *A. borneensis* (8.5, 13.0, 35.5 mm), and *A. bicolor* (31.3-49.2 mm) were collected from the Celebes Sea and Tomini bay. Collection of smaller leptocephali (8.5 and 13 mm) of *A. celebesensis* (13.0-47.8 mm) and *A. borneensis* indicated that these species spawn in the Celebes Sea.

Aoyama et al. (2003) found that these species were found to have short-distance spawning migration. Wouthuyzen et al. (2009) reported that seasonal spawning for *A. celebesensis* in Tomini bay was in late February and early May. Further, it was also stated that spawning of this species stopped for a several-month period (late summer and fall) each year, attributed to the dry season on Sulawesi Island as controlled by the regional tropical monsoon cycle. In addition, *A. borneensis* wherein 8.5 mm leptocephali were also found to spawn in close proximity with *A. celebesensis* in the Celebes Sea (Aoyama et al., 2003; Kuroki et al., 2006).

These species have different larval durations and may recruit at different times and locations. *Anguilla marmorata*, *A. japonica*, and *A. luzonensis*, which were believed to have spawned with the NEC region, were carried by the NEC and recruit to Taiwan and the Philippines. *A. marmorata* is an abundant species in Taiwan, while *A. bicolor pacifica*, *A. japonica*, and *A. luzonensis* occur at very low proportions (Han et al., 2012; Leander et al., 2012). These species, including *A. luzonensis* and *A. celebesensis* were also found to recruit in the Philippines. In the northern part of the Philippines (Cagayan), *A. marmorata* is an abundant species that may vary due to seasonal changes. Other species, i.e., *A. bicolor pacifica*, may occur at a higher percentage (Aoyama et al., 2015). Moreover, *A. luzonensis* were found to continuously recruit in Luzon at similar proportions (30-40%) in the two-period study. On the other hand, *A. japonica* and *A. celebesensis* were rarely recruited in the northern part of the Philippines (Aoyama et al., 2015). The southern part of the Philippines, Mindanao Island, is composed of almost a similar species. *A. marmorata* (76.1%), which was also an abundant species followed by *A. bicolor pacifica* (19.5%). *A. celebesensis* (3.0%), and *A. interioris* (1.1%) also recruit at low percentages (Shirotori et al., 2016). At the same time, *A. luzonensis* (n=4) and *A. borneensis* (n=1) were found to rarely recruit in the area. Hence, abundance of *A.*

marmorata and *A. bicolor pacifica* were already documented in the northern and southern part of the Philippines and *A. luzonensis* being the second most abundant species in the north.

1.5 Lagonoy Gulf, Philippines

Species composition studies were only limited to the northern and southern parts of the Philippines. However, no studies were carried out in the mid-part of the country facing the pacific ocean and located near the NEC bifurcates, where Bicol Region is located. One of the largest (3,701 km^2) and most important fishing grounds in the Bicol Region is Lagonoy Gulf, located in the northeastern part of the Philippines (Olaño et al., 2018; Soliman, 2013). It is bordered by three provinces, namely Albay, Camarines Sur, and Catanduanes. One of the important oceanographic features found in the area is the NEC bifurcates (14.2°N), and one of the current traverses north of the Philippines to Taiwan and Japan, known as the Kuroshio current (Qu & Lukas, 2003). Therefore, its location, near the spawning ground of freshwater eels, is geographically advantageous for recruiting glass eels. However, there are no available data on its species composition.

1.6 Dissertation outline

Anguilla marmorata and *A. bicolor pacifica*, the dominant species in Cagayan and Mindanao, are being exported to the eel consuming countries in the East Asia. A larger percentage of *A. bicolor pacifica* is exported to eel consuming countries in East Asia, hence higher-value than *A. marmorata*. Although *A. marmorata* is the most abundant species in the Philippines, its market value is lower than that of *A. bicolor pacifica*. In addition, *A. luzonensis*, one of the most abundant species in Cagayan, is also being exported, yet there is no information on its current market value. Therefore, the value

addition of *A. marmorata* and *A. luzonensis* by utilizing these commodities into products that can compete in the Philippine market could be done However, there is no available information on the fishery of Anguillids in Lagonoy Gulf; hence this study was conducted.

Chapter 2 introduces the freshwater eel fishery in the tributaries along Lagonoy Gulf. Information on the consumption and gears used for collection. The species composition of glass eels was reported based on the morphological identification limited to pigmentation patterns.

Chapter 3 clarifies the proportion of Anguillid species recruited in the tributaries along Lagonoy Gulf using molecular tools. In addition, the population structure of *A. luzonensis* was also investigated.

Chapter 4 clarifies the pigmentation pattern useful for identifying *A. marmorata*, *A. luzonensis*, and confirmation for *A. bicolor pacifica*. Pigmentation patterns of several individuals identified by the molecular analysis were revisited for clarification.

Chapter 5 investigated the gelatin content of soup of *paksiw* made from *A. marmorata* and *A. bicolor pacifica*. The presence of the gelatin and the estimated protein contents were compared between *A. marmorata* and *A. bicolor pacifica paksiw* soup.

Chapter 6 includes the summary of the results, implications, and the future direction of this study.

Chapter 2 Freshwater Eel Fishery in the Tributaries along Lagonoy Gulf

Abstract

Anguilla marmorata, *A. bicolor pacifica*, *A. japonica*, *A. bicolor bicolor*, *A. luzonensis* and *A. celebesensis* found in the Philippines were reported to have spawned in the North Equatorial Current with Kuroshio Current as one of the factors influencing its migration. With Bicol Region located near the Kuroshio Current, Anguillid recruitment is possible, yet no available data on its consumption, collection, and species composition. Therefore, in this study, the Anguillid consumption and collection have been documented through key informant's interview. In addition, the species composition of Anguillids was investigated using morphological identification limited to pigmentation pattern. Freshwater eels caught are mainly cooked using indigenous recipes such as grilling (*inihaw*), with vinegar (*paksiw*), and with coconut milk (*ginataan*) consumed at home during special occasions. Collection of adult freshwater eels uses active gears such as hook and line, speargun, push net, and electrofishing gear; and rock mounds and fish traps which are passive gears. On the other hand, modified fyke net and scoop net is commonly used for glass eel collection. Glass eel specimens collected from 2018-2019 from Comun river, Albay; Lagonoy river, Camarines Sur; and Bato river, Catanduanes were identified based on the morphology using the pigmentation pattern based on an illustration. *Anguilla japonica* was easily identified by the absence of pigmentation. *Anguilla bicolor pacifica* seems to be almost identified by the pigmentation pattern on the tail reaching the caudal fin tip. However, *A. mamorata* and *A. luzonensis* was difficult to distinguish; hence all individuals with pigmentation pattern not reaching the tail tip were grouped under *A. marmorata.* Species composition showed that *Anguilla marmorata* was the dominant species found in all the rivers (82.3-99.7%). *Anguilla bicolor pacifica* was the second

abundant species in Comun (12.4%) and Lagonoy (17.5%) rivers, whereas rare in Bato (1.0%) rivers. *Anguilla japonica* was found rare in Comun (0.1%) and Lagonoy (0.3%) rivers. Due to the difficulty in distinction of *A. marmorata* and *A. luzonensis*, further study on the confirmation of the species identification and the species composition by molecular analysis is necessary. Precise information on the species composition is important for the effective management and utilization of Anguillid resources in the region.

2.1 Introduction

The Philippines is a fish-consuming country with a mean 1-day per capita fish and fish products consumption of 36.8 kg per year in 2015 (DA-BFAR, 2018). Fish is considered Philippines's staple food next to rice (Philippine Statistics Authority (PSA), 2016). However, freshwater eel consumption is not that popular; hence the market for adults in the Philippines is not established. On the other hand, there is a high demand for glass eels and elvers driven by the East Asian market (Crook, 2014; Cuvin-aralar & Romana-eguia, 2019).

The Philippines has been identified as an important source of Anguillids in recent years (Crook, 2014). *Anguilla marmorata*, *A. bicolor pacifica*, *A. japonica*, *A. bicolor bicolor*, *A. luzonensis* and *A. celebesensis* were species reported to recruit in the Philippines (Aoyama et al., 2015; Han et al., 2016; Shirotori et al., 2016). Mainly, freshwater eels were collected from Cagayan and have expanded to Mindanao Anguillids recruited in the north and southern part of the Philippines were presumed to have spawning grounds in the NEC (Han et al., 2012, 2016; Kuroki et al., 2009; Kuroki et al., 2012; Miller et al., 2002; Tsukamoto et al., 2002). One of the NEC bifurcates, the Kuroshio Current, influences the migration of glass eels in the Philippines to Taiwan and Japan (Han et al., 2016; Leander et al., 2012, 2013). Kuroshio Current is an important oceanographic feature found in Lagonoy Gulf, Bicol Region (Olaño et al., 2018; Soliman, 2013); hence, there is a great possibility for freshwater eel resources in the Gulf.

River deltas are the usual glass eel collection sites, whereas the upstream portion of the river is the fishing ground for adult eels. Nieves et al. (2021) reported 13 potential eel migration and settlement sites in Albay, Camarines Sur, and Catanduanes. In Albay, migrating eels can settle into inland bodies of water via three major rivers, which include Comun/Balza River in Malinao, Malilipot River in Malilipot, and Bacacay River in the

municipality of Bacacay. In Camarines Sur, Sagñay river in Sagñay and Pili river in Presentacion have been known as eel fishing areas and settlement sites for species with faster metamorphosis because of their direct access to Lagonoy Gulf. The Catanduanes the river system, the Bato and Panganiban rivers, the Pajo river in Virac and Mayngaway river in San Andres, are most advantageous for migrating freshwater eel seeking to settle in freshwater habitat due to proximity to the Pacific Ocean where the known Anguillids spawning ground in the western waters of Marianas is located, hence, the higher probability for migrating freshwater eels to settle in the rivers and tributaries of Catanduanes.

Freshwater eels at the glass eel stage are commonly collected using a fyke net installed vertically hanged in the water, with its bottom edge held down by weights and its top edge buoyed by floats (Muthmainnah et al., 2016). In Cagayan, fyke nets are operated in two ways; the first one is fixing the nets in a stationary position on the sides of the riverbank, and the other way is operating the nets by dragging like a trawl. The fyke net is fixed at night, and then the collection of the trapped elvers is done in the morning (Ame et al., 2013). The fence net, which is also used for glass eel collection, is made of a small mesh-size net and two wings with a wooden stick to keep the wings and the mouth standing; set at the sides of rivers for the whole day and hauled four times a day (Suryati et al., 2019). The scoop net, made of wooden sticks and covered with a small mesh-size net, is also for glass eel collection set in the mouth of rivers and operated during the night for 3 hours (Suryati et al., 2019).

Nieves and Nolial (2019) reported that the collection and post-harvest handling practices for glass eels in Lagonoy Gulf have been established based on the techniques and experiences from the milkfish (*Chanos chanos*) fry industry and the techniques brought by consolidators and other buyers outside the region. These steps included (1)

sorting to remove debris and other species; (2) conditioning by stocking the larvae in a container with freshwater at specific density; (3) prophylactic treatment of Methylene blue and aerated for 24hours; (4) sorting & culling, to remove weak larvae; (5) packaging with the use of 1/3 filled plastic bag and inflated with 2/3 oxygen; (6) a small amount of methylene blue is again poured in the water in a separate cool tub prepared and about three (3) eel fry bags placed inside with cube ice over it and (7) transport done early in the morning or late in the afternoon. A typical post-handling harvest practiced along Lagonoy Gulf is the "rapid salinity shocking," wherein sorted glass eels are directly transferred to containers with freshwater, apparently eliminating potential pathogens resulting from the change in salinity.

Anguilla marmorata, *A. japonica*, *A. luzonensis*, *A. bicolor pacifica*, and *A. celebesensis* was believed to have spawning ground in the North Equatorial Current (Han et al., 2012, 2016; Kuroki et al., 2009, 2012; Tsukamoto, 1992). However, these species distribution and spawning season was reported to be different (Han et al., 2012, 2016; Leander et al., 2013). *A. marmorata*, *A. japonica*, and *A. luzonensis* were found to recruit in Taiwan, northern and southern Philippines at different proportions which may vary depending on the season or months (Aoyama et al., 2015; Han et al., 2012; Leander et al., 2012; Shirotori et al., 2016; Yoshinaga et al., 2014). Commonly, *A. marmorata* was found dominant in Taiwan, Cagayan and Mindanao (Aoyama et al., 2015; Shirotori et al., 2016).

Adult eels commonly inhabit the upstream of freshwater systems (Moriarty, 2003). Adult eels were of lower value than the glass eels (Crook, 2014). Yellow eels, the non-matured adult eels, were reported to be traded in Japan, Taiwan and Korea based on a survey conducted in Luzon and Mindanao (Southeast Asian Fisheries Development Center (SEAFDEC), 2019). Reports in the catch and production of yellow eels were not separated from elvers. In 2017, 0.64 tonnes of elvers/yellow eels were caught Luzon and

Mindanao and about 20.23 tonnes were produced by eel farms in Luzon wherein few of these catch and production were for local consumption (SEAFDEC, 2019). Seine net, bamboo trap, hook line and speargun were used for catching elvers/yellow eels (SEAFDEC, 2019). There were two company based in the Philippines (Davao and Metro Manila) who offer adults eels online (Crook, 2014). Commonly, freshwater eels (*Igat*) were caught only for home consumption in the Isabela, Cagayan and Marikina, Philippines (Foundation for the Philippine Environment (FPE), 2009; Geronimo et al., 2016; Magulod Jr., 2018).

Morphological identification using pigmentation pattern have long been practiced by fishers in the field setting, however, this had been considered a problem by fishers due to the difficulty in distinction of Anguillid species at the glass eel stage (SEAFDEC, 2019; personal communication). The dominance of *A. marmorata*, frequent recruitment of *A. bicolor pacifica*, presence of *A. luzonensis* and rarity of *A. japonica* were documented in the north and south parts of the Philippines (Aoyama et al., 2015; Shirotori et al., 2016), and no study in the mid-part of the Philippines. Unfortunately, in Lagonoy Gulf where the geographic location is advantageous for Anguillid recruitment, no available data on its consumption, collection, and species composition, hence, the relevance of the present study. Species of glass eel recruiting in Lagonoy gulf were identified using caudal pigmentation pattern to describe the species composition.

2.2 Materials and Methods

2.2.1 Interview

Data on consumption and fishing gears was collected using Key Informant Interview (KII) involving 37 key informants composed of eel collectors or fishers and

consolidators. The questionnaire was crafted to elicit information mainly on consumption and catch of freshwater eels (Appendix 1).

2.2.2 Study site and sample collection

Glass eels were collected from three main rivers, namely the Comun river (13°25'09.1" N 123°42'44.6" E) in Albay, the Lagonoy river (13°43'37.9" N 123°35'11.0" E) in Camarines Sur, and the Bato river (13˚35'40.0" N, 124˚17'10.0" E) in Catanduanes (Fig. 2.1).

The glass eels were collected using a modified fyke net that was 1.15 m in height (H) and 10 m in length (L), with 2-8 m long wings and a 2.5 m long catching bag [oval-shaped opening dimensions - 1.15 m H and 3 m width (W)] installed near or at a 3-4 m distance from the river mouth at a water depth of 1-1.2 m in the Comun river and Lagonoy river. A push net with dimensions 1.0 m H by 1.15 m W was positioned at a 3-4 m distance from the river mouth at a water depth of 1-1.2 m in the Bato river.

A one-day sample collection was conducted beginning at 18:00 h for 2-4 h during the new moon phase between August 2018 and June 2019 in the Comun, and Bato rivers; and July 2018 and June 2019 in the Lagonoy river. If the number of samples collected was more than 300, random selection was carried out (Nieves et al., 2021).

Excess glass eel specimens were returned to the river for resource conservation as 300 individuals were enough to estimate the species composition (Nieves et al., 2021). The collected glass eels (n=4,801) were preserved in 95% ethanol for subsequent analysis.

2.2.3 Morphological observations

The tail part of the glass eel was observed under a stereomicroscope (2x magnification) with an external light source and then photographed. The pigmentation

patterns on the tail and the caudal fin were used for morphological identification of the species (Table 2.1).

2.2.4 Species composition

Proportion of the species recruited in each of the rivers were expressed as the percentage of a single species over the total number of freshwater eels collected for each of the sites.

2.3 Results

2.3.1 Consumption and collection of freshwater eels

Adult freshwater eels, locally known as *kasili*, *burirawan* (*A. marmorata*) and *butla* (*A. bicolor pacifica*) are caught in the rivers and rice field irrigation systems for home consumption and during special occasions such as birthdays, fiesta and other family gatherings. It is traditionally cooked using indigenous recipes such as grilling (*inihaw*), with vinegar (*paksiw*), and with coconut milk (*ginataan*). In grilling, eels are marinated in vinegar and soy sauce with spices like garlic and pepper. *Paksiw* is prepared by stewing eel in water with vinegar added with ginger, garlic, onions, and pepper. It is also cooked with coconut milk, garlic, onion, ginger, and pepper. Sometimes, this recipe is added with hogplum leaves (*lubas*). In addition, freshwater eels are also consumed fried. However, adult freshwater eels are being marinated first with a small amount of vinegar with salt before frying.

Eel fishing gears consist of active and passive gears usually made of traditional (i.e., bamboo and rattan) and modern netting materials. Hook and line (*banwit*), consists of a combination of lines and hook (with single or multiple hooks) with natural baits (Fig. 2.2a). Speargun (*pana*) is designed to launch a spear at fish, powered by the rubber (Fig.

2.2b). The basic components are a spear (75-80 cm), a stock or barrel, and a handle or grip containing a trigger mechanism. *Piltik* is a local version of the speargun (Fig. 2.2c) operated by an individual using spear with 2 or more prongs (1m in height). The basic components are prong, rubber, and bamboo with a flashlight as an accessory during night operation. Prongs used in spears are made of iron nails or pointed metal rods. The operation is similar to speargun but typically used in shallow water areas. Push net, locally known as *sud-sod*, is a gear that measures 1.0m (H) x 1.15m (W), with different shapes (circular & rectangular), sizes, and materials, and generally made of split bamboo and steel bar (Fig. 2.2d). Normally, the operation is daytime in shallow waters or even turbid waters thus, disturb organisms in the river system. Electro-fishing gear is made from assembled battery pack containing a high voltage current used to electrocute fishes (Fig. 2.2e). It consists of a battery, wire, metal, bag/box, scope net, and bamboo or pipe.

Fish traps (*bubo*) is a passive gear that occurs in different shapes and sizes made of split bamboo separated by narrow interspaces braided together by the strands of cane (Fig. 2.2i). Traps may have single or multi-opening, inwardly projecting bamboo splits permitting entry of fish without any means to escape. Traps are placed underneath the water with or without baits and normally set up at night, and the catch is harvested the following morning. Bamboo filter trap, locally known as *ansag* is an indigenous trap built for volume fishing (Fig. 2.2i) designed to take advantage of the high waters during the rainy season. However, the bamboo filter traps eels and other fish species, allowing the fishers to wait and only handpick the catch conveniently. Rock mounds (locally known as *patambak*, *hilay* in Albay; *gango* in Cam Sur), an aggregating device, use stone piles with a net flooring that is usually 0.5 to 1.3 m high depending on the water depth at the lowest tide (Fig.2.2h). This type of gear is usually set in the morning in the shallow-stony water area and harvesting done in the following day or after a week by raising the four

tips of the net flooring, and the stone removed slowly to concentrate the fish into the net. Ichthyotoxic plants such as *tuba-tuba* (*Jatropha curcas*), *tubli* (*Derris* spp.) and *bayati* (*Anamirta cocculus* Linn.) are plants which have certain toxicity to fish. The fruits, roots or its stems are mashed, and the extract are poured in a certain water area to stupefy fish for easy catching.

The modified fyke net (MFN) known locally as *kubong*, is a main collecting gear for glass eel (Fig. 2.2f) with a two-8m long wings 1.15m (H), 10m (L) with a 2.5m catching bag and at least 7 bamboo poles as support. Commonly, they are set in two ways – the stationary and then by dragging and is operated by 2-3 fishers from 6:00 PM onwards during new moon to first quarter. Scoop net (Fig. 2.2g) is used to collect harvest trapped in the catching bag or funnel.

2.3.2 Morphological observations

Pigmented and non-pigmented glass eels were observed based on the illustration (Table 2.1). Among the four illustrations corresponding to four species, very few individuals were observed having no caudal pigmentation and was identified as *A. japonica*. Many individuals were identified as *A. bicolor pacifica* based on the pigmentation pattern that extend until the tip of the tail. Almost all the glass eels had pigmentation not reaching the tip of the caudal fin which maybe *A. marmorata* or *A. luzonensis*. Background coloration on the tail without scattered pigments; a uniform pigment patches on the tail and gradations of pigment patches on the posterior to the anterior part of the tail were also observed; however, these patterns were not found in the illustration for identification of glass eel species (Table 2.1). In addition, a gray-to-black line on the tail also made distinction by pigmentation pattern among species challenging.

2.3.3 Species composition

Based on the assumption of *A. marmorata*, *A. bicolor pacifica*, and *A. japonica* recruitment, the species composition yielded an aggregate percentage of 89.8%, 10.1%, and 0.1%, respectively. *A. marmorata* was the dominant species found in Comun (87.5%), Lagonoy (82.3%), and Bato (99.7%) rivers (Fig. 2.2). *A. bicolor pacifica* was the second abundant species in Comun (12.4%) and Lagonoy (17.5%) rivers whereas rare in Bato (0.3%) river (Fig. 2.2). *A. japonica* was found rare in Comun (0.1%) and Lagonoy (0.3%) rivers (Fig. 2.2a,b).

2.4 Discussion

2.4.1 Consumption and catch of freshwater eels

Among the cooking way for freshwater eels, grilling and *paksiw* are known in the Philippines as a cuisine utilizing fish and meat. The use of coconut milk in cooking is quite famous for Bicolanos. Currently, there is no standard way of cooking freshwater eels in the Philippines. Consumption of freshwater eels may have existed a long time ago. However, knowledge of its consumption and cooking was not successfully preserved and transferred to the younger generation.

Adults and glass eels are collected using different gears which can be classified as active or passive wherein active gears. Active gears such as hook and line, spear, spear gun, push net and electrofishing gear are designed to chase and capture target and non-target species singly or in combination with bait or ichthyotoxic plants. Among the active gears used, electrofishing gear is illegal and destructive to all aquatic organisms within the radius of the electrical field. Mainly, these active gears are used to catch adult eels. On the other hand, rock mounds and traps such as *bubo* and *ansag* were passive gears for collection of adult eels. In addition, the trap catches various fish species filtered from the

trap into the traditional and intricate structure that allows the fishers to wait for fish to arrive and be handpicked conveniently. In the case of ichthyotoxic plants, these are used before using an active gear such as spear gun to kill, stupefy, disable, or fish and other aquatic animal render unconscious or stunned before catching.

Most common gear used for glass eel collection is the fyke net and scoop net which mostly stationary than by dragging. Once the fyke net traps glass eels, a scoop net is used to harvest the trapped eels. Although push net is used for adult fishing, this gear had also been used for glass eel collection specifically in Catanduanes.

2.4.2 Morphological observations

Based on the pigmentation pattern illustration (Table 2.1), *A. japonica* can easily be distinguished. In addition, *A. bicolor pacifica* also seemed to be almost distinguished. However, *A. marmorata* and *A. luzonensis* was difficult to distinguish due to the similar pigmentation patterns with based on the description (Table 2.1) The rigidity and not scattered inner pigmentation pattern based on the description (Table 2.1) of *A. luzonensis* was too difficult to distinguish from *A. marmorata*. *Anguilla marmorata*, *A. luzonensis* and *A. celebesensis* which have been found to recruited in Cagayan, were reported to have similar pigmentation patterns (Aoyama et al., 2015; Yoshinaga et al., 2014). However, the description of illustration used to identify Anguillids clearly separates *A. marmorata* from *A. luzonensis* (Table 2.1). No reports have discussed the pigmentation patterns that may distinguish *A. celebesensis* from *A. marmorata* and *A. luzonensis*. Anguillids catch in Cagayan is comprised of *A. marmorata*, *A. bicolor pacifica*, *A. luzonensis* and *A. celebesensis* wherein *A. marmorata* is the most dominant and *A. luzonensis* as constantly recruiting species (Aoyama et al., 2015), which may also recruit in Lagonoy Gulf given the advantage of geographic location, hence identification based on pigmentation pattern

must be validated. Although the illustration showed that *A. marmorata* and *A. luzonensis* can be distinguished, distinction based on our observation was difficult. Though occurrence of *A. luzonensis* and *A. celebesensis* might be possible, with the abundance of *A. marmorata* in the Philippines (Aoyama et al., 2015; Shirotori et al., 2016), we have grouped all specimens with pigmentation pattern not reaching the caudal fin tip as *A. marmorata*. With the abundance of *A. marmorata* in the Philippines, our observation that *A. bicolor pacifica* can be distinguished and identification of *A. japonica*, we have provisionally described the proportion of these three species.

2.4.3 Species composition

Proportion of the Anguillids recruited in Lagonoy Gulf was reported based on the *A. marmorata*, *A. bicolor pacifica* and *A. japonica*. Among these species, *A. marmorata* was found dominant across all the sampling sites, with Bato river having the highest percentage (99.0%, Fig. 2.2). In Bato river, *A. bicolor pacifica* occurred at a lower percentage than *A. marmorata* (Fig. 2.2c). On the other hand, *A. bicolor pacifica* is the second frequent species to occur in Comun and Lagonoy rivers (Fig. 2.2a,b). *Anguilla japonica* was found to rarely recruit in Comun and Lagonoy rivers (Fig. 2.2a,b) and was not found in Bato river (Fig. 2.2c).

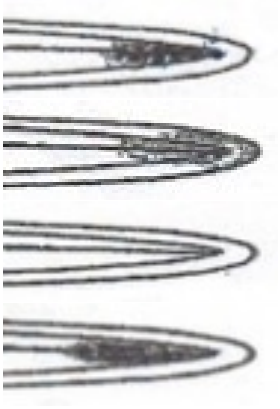
Based on the abundance of *A. marmorata*, *A. luzonensis* as the second dominant species and rarity of *A. celebesensis* in Cagayan (Aoyama et al., 2015), we speculated that *A. marmorata* is also abundant in Lagonoy Gulf. However, the data we reported for *A. marmorata* might also contain *A. luzonensis* and *A. celebesensis*. *Anguilla marmorata* and *A. luzonensis* were reported to have spawning sites in the NEC and experience similar oceanographic features (Aoyama et al., 2015; Han et al., 2016). Considering the spawning ground of *A. marmorata* and *A. luzonensis* in the NEC, which bifurcates near Lagonoy

gulf, recruitment of *A. marmorata* and *A. luzonensis* in the tributaries along Lagonoy Gulf is highly possible.

Anguilla bicolor pacifica is the second frequent species in Cagayan (period II) and Mindanao (Aoyama et al., 2015; Shirotori et al., 2016), which might also be similar in Lagonoy Gulf. Moreover, according to van Herwaarden, 2003, *A. bicolor pacifica* inhabits the Southern part of Luzon, the Visayas, and Mindanao, hence can be found in Lagonoy Gulf. In the case of *A. japonica*, rare occurrence were reported in Cagayan and Mindanao (Aoyama et al., 2015; Shirotori et al., 2016) similar to our observation in Lagonoy Gulf.

Under field setting and with the urgency in distinguishing the species at the least possible cost for the gatherers/collectors, morphological characters by pigmentation pattern seem to be the most practical method, which is practiced in Cagayan and Mindanao, where eel fishery is more established than in Lagonoy Gulf. However, among *A. marmorata* and *A. bicolor pacifica*, and *A. japonica* reported to occur in Lagonoy Gulf, distinction between *A. marmorata* and *A. luzonensis* using pigmentation pattern was difficult. Hence, a further study on confirmation of the species identification and composition using molecular analysis is deemed necessary.

Table 2.1 Guide used for species identification of glass eels by pigmentation patterns observed in the tail and caudal fin (Han, personal communication; Han et al., 2012; Shirotori et al., 2016).

Species	Caudal pigmentation description	Illustration
A. marmorata	With caudal pigmentation but not touching the end/tip of the tail; inner pigmentation is scattered	
A. bicolor pacifica	With caudal pigmentation that extends to the tip/end of the tail	
A. japonica	No caudal pigmentation	
A. luzonensis	The same with *A. marmorata*, however, inner pigmentation are rigid and not scattered	

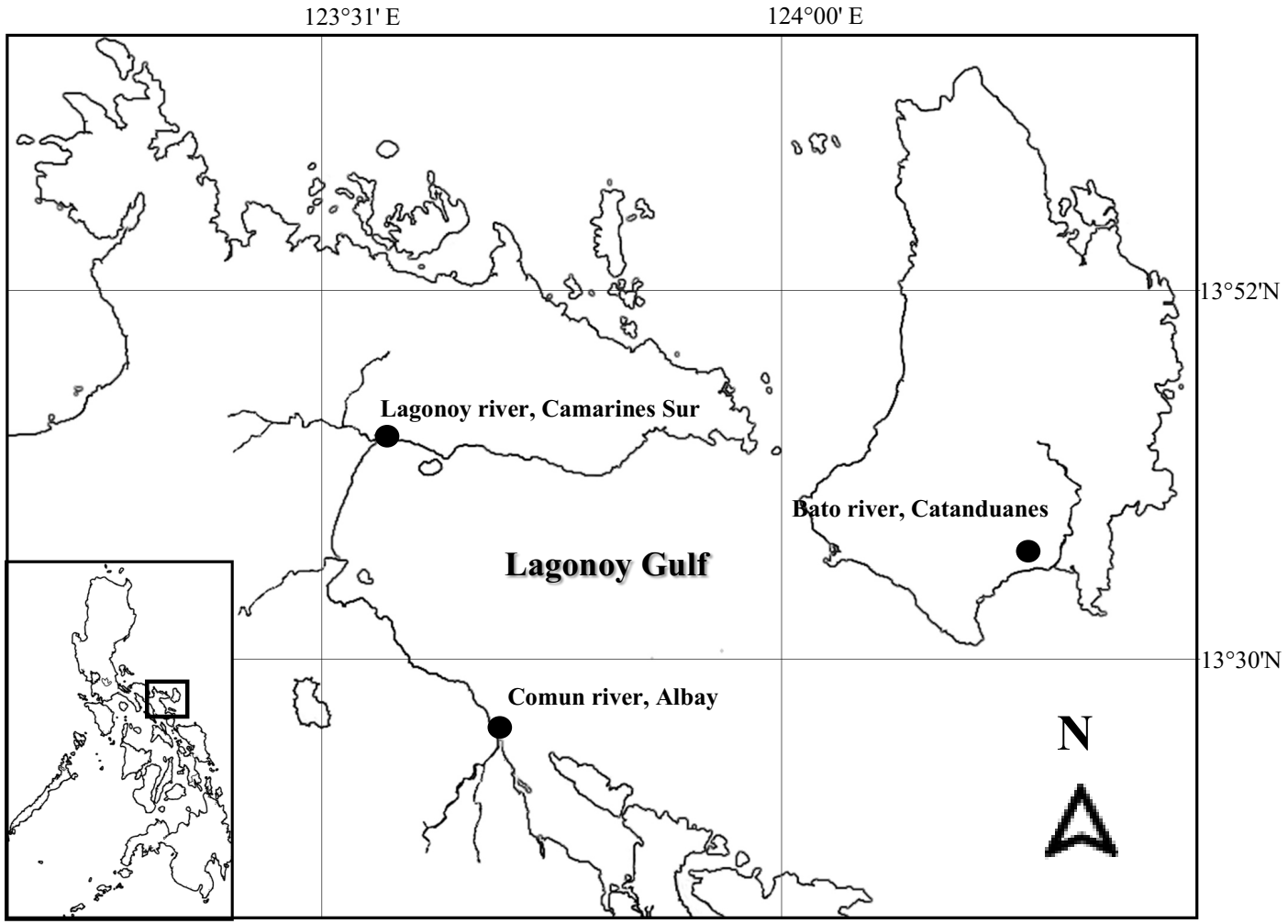


Fig. 2.1 Map showing the study area in Lagonoy Gulf, Philippines.
●, indicates the sampling sites in Albay, Camarines Sur and Catanduanes.

Fig. 2.2 Gears used for catching of freshwater eels.
(**a**) Hook and line (*banwit*); (**b**) spear gun (*pana*); (**c**) spear (*piltik*); (**d**) push net (*sudsod*); (**e**) electro-fishing gear; (**f**) modified fyke net (*kubong*); (**g**) scoop net; (**h**) rockmounds (*patambak/hilay*); (**i**) bamboo traps (*bubo*); (**j**) bamboo filter trap (*ansag*).

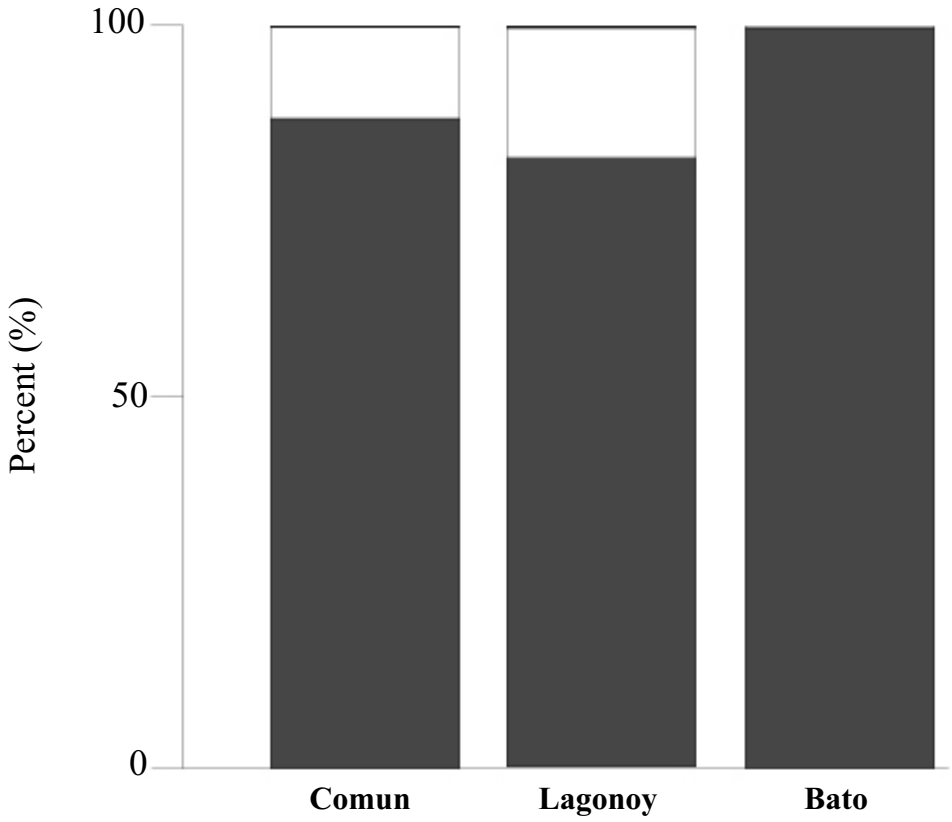


Fig. 2.3 Species composition of freshwater eels recruited in Comun, Lagonoy and Bato rivers.
■, *A. marmorata*; □, *A. bicolor pacifica*; ■, *A. japonica*

Chapter 3 Species Clarification of Pigmented Anguillid Glass Eels Collected from the Tributaries along Lagonoy Gulf, Philippines

Abstract

The pigmented glass eels *A. marmorata*, *A. luzonensis*, *A. bicolor pacifica and A. celebesensis* were found in the north and south of the Philippines; hence recruitment of these species in Lagonoy Gulf is possible. However, the provisional proportion of pigmented glass eels in Lagonoy Gulf that we have reported (Chapter 2) is only for *A. marmorata* and *A. bicolor pacifica* due to difficulty in distinction of these two species using pigmentation pattern. Therefore, in this study, we have clarified the species composition of pigmented Anguillids recruited in the tributaries along the Lagonoy Gulf, Philippines using molecular tools. Glass eel specimens were collected in 2018–2019 from the Comun river, Albay; the Lagonoy river, Camarines Sur; and the Bato river, Catanduanes. *Anguilla marmorata* was the most dominant species in all tributaries (71.1–98.0%). *Anguilla luzonensis* was first reported in Lagonoy Gulf using molecular analysis. *A. luzonensis* was the second most abundant species in the Comun and Lagonoy rivers (9.5 and 22.4 %, respectively). In the Comun and Lagonoy rivers, *A. bicolor pacifica* was the third most abundant species (7.7 and 6.5%, respectively). In Bato river, both *A. luzonensis* and *A. bicolor pacifica* occurred at relatively low percentage (1.0%). In addition, *Anguilla celebesensis* was to rarely occur only in Comun river (0.9%). *Anguilla luzonensis* collected from the Comun and Lagonoy rivers did not show a significant difference (F_{ST}=0.00825, $p>0.05$). This study provides important information for sustainable resource management and effective utilization of the eel species in these regions.

3.1 Introduction

The recent study on Anguillid species in the tributaries of the Lagonoy Gulf, Bicol reported the dominance of *A. marmorata*, followed by that of *A. bicolor pacifica*, and a negligible quantity of *A. japonica* based on morphology limited to pigmentation pattern (Nieves et al*.,* 2021). Although the illustrations for *A. marmorata* and *A. luzonensis* showed that these species have pigmentation, they were difficult to identify. Consequently, among more than 4,000 pigmented glass eels observed, 89.8% were grouped as *A. marmorata* and 10.1% as *A. bicolor pacifica*. In addition, *A. luzonensis* might also be found in the grouped *A. marmorata* individuals yet needed to be confirmed.

Anguilla luzonensis was discovered in Cagayan and described as a new species in 2009 using adult specimens (244-682 mm) (Watanabe et al., 2009). *Anguilla luzonensis* has potential as a fishery product since trade to eel-consuming countries in the East Asia has been reported (Ame et al., 2013; United Nations & United Nations (UN), 2016; Yoshinaga et al., 2014). Information on its population dynamics, stock status, and utilization is limited (Gollock et al., 2018). Due to the limited known range of *A. luzonensis*, it was recently assessed by CITES as a 'Vulnerable' (VU) species under category D2 (Gollock et al., 2018). Therefore, resource management strategies in support of effective utilization, economic potentials, and sustainability of *A. luzonensis* bioresources are needed.

Anguilla luzonensis may be widely distributed in the Western Pacific with reports of its occurrence in Cagayan, Mindanao, Taiwan and Okinawa, Ryuku archipelago (Aoyama et al., 2015; Han et al., 2012, 2016; Jamandre et al., 2007; Kita et al., 2021; SEAFDEC, 2019; Yoshinaga et al., 2014). *Anguilla luzonensis* is presumed to have spawned in the North Equatorial Current (NEC) (Han et al., 2016; Kuroki et al., 2012). One of the NEC bifurcates, the Kuroshio Current (Qu & Lukas, 2003), transported *A.*

luzonensis to Eastern and Northern Luzon and Taiwan (Han et al., 2016). *Anguilla luzonensis* was presumed to have similar spawning area in the North Equatorial Current and experience the same oceanographic conditions with *A. marmorata* (Aoyama et al. 2015; Han et al. 2016). As *A. luzonensis* specimens were found in the Cagayan and Mindanao waters (Watanabe et al., 2009; Aoyama et al., 2015; Shirotori et al., 2016), it can be speculated that this species might also occur in the Lagonoy Gulf. In addition, the Bicol region faces the Kuroshio Current, hence highly possible that these species may also occur in the Lagonoy Gulf in the same region. The Bicol region ranked 2nd for highest municipal fish production (DA-BFAR, 2019). Therefore, Anguillid glass eels might be one of the important target species for fishermen, but limited fishery data are available. For resource management, the precise species composition must be clarified. The use of morphological identification limited to pigmentation pattern alone was not enough to distinguish the pigmented Anguillid species; hence, molecular identification may be used for precise species identification and confirmation of species composition.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), a fast and reliable technique, could identify 14 known *Anguilla* species (Aoyama et al., 2000; Lin et al., 2002; Watanabe, 2003; Watanabe et al., 2005) among the 18 known species and subspecies. Although *A. bicolor* subspecies cannot be distinguished, PCR-RFLP technique was suggested to be refined using a large number of samples to identify any eel species (Watanabe et al., 2003; Watanabe et al., 2005). There may be cases where PCR-RFLP could not identify a specimen; hence, DNA sequencing may be used for further species identification (Aoyama et al., 2000). The DNA sequences were also used in investigating molecular evolution (Aoyama et al., 2001; Minegishi et al., 2005). Thus, *A. luzonensis*, *A. marmorata*, *A. bicolor pacifica*, *A. bicolor bicolor*, *A. japonica*, and *A.*

celebesensis, commonly found in Luzon, Philippines, may be identified by PCR-RFLP and DNA sequencing analysis.

In this study, the precise species composition of glass eels recruiting in the tributaries along the Lagonoy Gulf was clarified using we molecular tools. *Anguilla luzonensis* population structure was also confirmed.

3.2 Materials and Methods

3.2.1 Glass eel specimens

The specimens analyzed in this study were glass eels that were identified on the basis of morphology (Nieves et al., 2021; Chapter 2) collected during August 2018 and August 2019 in the Comun river, July 2018 and July 2019 in the Lagonoy river, and December 2018 and July 2019 in the Bato river. Among the morphologically identified pigmented glass eels, approximately 10% (n=554; Comun=220, Lagonoy=232, Catanduanes=102) were randomly selected for molecular analysis.

3.2.2 DNA Extraction

We cut 25 mg of the middle-part of a glass eel into small pieces and lysed these overnight. We extracted DNA using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) or FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., MI, USA). Extracted DNA was quantified using a NanoVue Plus spectrophotometer (GE Healthcare).

3.2.3 PCR amplification

The cytochrome c oxidase 1 (COI) target fragment gene was amplified using the designed primers 5503F1 (5'-CCGCTTAAACATTCAGCC-3') and 7138R1 (5'-

GGGGGTTCAATTCCTTCC-3'). The PCR reaction was performed in a 25 µl mixture containing 1.0 µl DNA template, 2.5 µl 10× buffer [magnesium (Mg^{2+}) plus], 2.0 µl 2.5 mM deoxynucleoside triphosphate (dNTP), 1.25 µl 10 µM each primer, 0.125 µl Taq polymerase (Takara Bio Inc., Shiga, Japan), and double-distilled water. Thermal cycler profile included initial denaturation of 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, elongation at 72 °C for 60 s, and final extension of 72 °C for 10 min. Two microliters (2.0 µl) of the PCR amplicons were visualized on 1% agarose Tris-acetate-EDTA (TAE) gel electrophoresis with GelRed Nucleic Acid Gel Stain (Biotium, CA, USA) (1:100 dilution with distilled water), viewed under UV transilluminator (Ultra-Violet Products) then photographed.

3.2.4 Restriction Fragment Length Polymorphism (RFLP) analysis

The simulation of restriction digestion was carried out for the target species *A. marmorata*, *A. bicolor pacifica*, *A. luzonensis*, *A. japonica*, *A. bicolor bicolor*, and *A. celebesensis* found in the Philippines. The complete mitochondrial genome of these six Anguillid species (Accession nos. AB469437, AP007242, AP007237, AP007236, AP007239, AB038556) was downloaded from the National Center for Biotechnology Information (NCBI) and aligned using MEGA X software (Kumar et al., 2018). Thirty-two restriction enzymes with clear and identifiable cleavage sites within the (16S ribosomal RNA (16S rRNA) and COI fragment genes were used for virtual digestion. Based on its ability to cut DNA into fragments and the patterns produced, *Msp* I was used for species identification. *Dde* I was used to distinguish *A. bicolor pacifica* from *A. bicolor bicolor* since *Msp* I cannot distinguish these two species. Restriction digestion was carried out in a 10 µl reaction mixture with 80-100 ng amplified product according to the *Msp* I and *Dde* I protocol (Takara Bio, Japan). The RFLP patterns were visualized in a 2%

agarose Tris-Borate-EDTA (TBE) gel electrophoresis with GelRed Nucleic Acid Gel Stain (Biotium, CA, USA) (1:3:6 dilution with loading buffer and distilled water) then photographed. Expected RFLP patterns for the specimens were used for species identification. The PCR-RFLP profiles of several samples were visualized in a lab-on-chip electrophoresis using the DNA1000 LabChips (Agilent Technologies, Inc.) with the 2100 Bioanalyzer microchip capillary electrophoresis system according to the manufacturer's instructions. The obtained RFLP patterns were analyzed using 2100 Expert software.

3.2.5 DNA sequencing and Phylogenetic analysis

The designed four oligonucleotide primers, 5511F2 (5'-ACATTCAGCCATCTTACC-3'), 6468R3 (5'-TGCRATGATTATTGTGGC-3), 6126F3 (5'-VCCAGTCCTAGCTGCAGG-3'), and 7131R2 (5'-CAATTCCTTCCTTTCTTG-3') were used to sequence the COI target fragment gene. The PCR product purified by Agencourt AMPure XP (Beckman Coulter, CA, USA) was used for direct cycle sequencing with the ABI BigDye v.3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA). Sequences generated from the 3130 Genetic Analyzer (Applied Biosystems, CA, USA) (Accession Numbers: LC588356- LC588371, LC588373-LC588374) were edited and manually aligned using Chromas version 2.6.6 and the MEGA X (Kumar et al., 2018) software, obtaining 1,431 bp after truncation. The two specimens that failed to show the high-intensity PCR bands required for RFLP were directly sequenced. A model test was conducted using MEGA X (Kumar et al., 2018) and the model with lowest Bayesian Information Criterion (BIC) score was selected. The Maximum Likelihood tree was constructed using the HKY+G+I model with *Stemonidium hypomelas* (NC013628) as an outgroup using MEGA X (Kumar et al., 2018) with 1,000

bootstrap probabilities for species identification of unknown RFLP patterns and confirmation of representative specimens with expected ones.

3.2.6 Mitochondrial DNA analysis

All (72 individuals) *A. luzonensis* partial COI target fragment genes were directly sequenced using the designed primer 6126F3 (5'-VCCAGTCCTAGCTGCAGG-3') by ABI BigDye v.3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA) and were manually aligned using Chromas version 2.6.6 and the MEGA X (Kumar et al.,2018) software, obtaining 663 bp after truncation. A Maximum Likelihood tree was constructed using T92 with *A. japonica* (AB038556.2) as an outgroup using MEGA X (Kumar et al., 2018) with 1,000 bootstrap probabilities to examine the geographical similarities between individuals and the phylogenetic relationship.

3.2.7 Statistical analysis

The genetic variability was characterized by the amount of nucleotide substitutions calculated by the ARLEQUIN 3.5.2.2 software (Excoffier & Lischer, 2010). The genetic diversity between rivers was investigated using the fixation index (F_{ST}) for all pairwise comparison (10,230 permutations) using the ARLEQUIN 3.5.2.2 software (Excoffier & Lischer, 2010). Only one *A. luzonensis* individual was identified in the Bato river, thus, was excluded in computations.

3.3 Results

3.3.1 Species identification

The simulated PCR-RFLP patterns of the target species *A. bicolor pacifica*, *A. bicolor bicolor*, *A.marmorata*, *A. japonica*, *A. luzonensis* and *A. celebesensis* after *Msp* I

digestion were determined (Fig. 3.1). *Anguilla bicolor pacifica* (lane A) and *A. bicolor bicolor* (lane B) have identical RFLP patterns. *Anguilla marmorata* (lane C) also has the same largest band (825 bp) as that in *A. bicolor pacifica* (lane A) and *A. bicolor bicolor* (lane B). Moreover, the doublet with sizes 340 bp and 370 bp may also facilitate the distinction of *A. marmorata* (lane C). *Anguilla japonica* (lane D), *A. luzonensis* (lane E), and *A. celebesensis* (lane F) also have the same size of the largest band (663 bp). There were two separate bands (289 bp, 370 bp) in *A. japonica* (lane D), doublet (340 bp, 370 bp) in *A. luzonensis* (lane E), and singlet (370 bp) in *A. celebesensis* (lane F). In addition, *A. japonica* (lane D) and *A. luzonensis* (lane E) has a singlet (163 bp), whereas *A. celebesensis* (lane F) has two bands (163 bp, 192 bp) between 100-200 bp. The shortest band with size 104 bp is common for all the species. The *Dde* I digestion (Fig. 3.1) shows different RFLP patterns that can be used to identify the six species. The largest band with different sizes from lanes A to F can distinguish *A. bicolor bicolor* (lane B), *A. marmorata* (lane C) from the other species. *A. bicolor pacifica* (lane A) and *A. japonica* (lane D) have nearly similar largest band. However, *A. bicolor pacifica* (lane A) may be distinguished from *A. japonica* (lane D) by the absence of approximately 350 bp band.

Fig. 3.2 shows the gel electrophoretic patterns of *Msp* I and *Dde* I digestion of the Anguillid specimens. The RFLP patterns of more than 300 bp bands were clearly observable, whereas the bands below 200 bp were not clearly visible. For *Msp* I, the largest bands of more than 800 bp were similar in lanes 1-5 and 8. A doublet was observed between 700-900 bp in lane 5. Lanes 6-7 have similar largest bands between 600-700 bp. The RFLP pattern in lanes 1-4 and 6-8 had similar thick bands between 300-400 bp with an intensity similar to that of the largest band. In *Dde* I, lanes 1-2 and 4-8 have the same RFLP patterns. Lane 3 has a largest band between 800 bp and 900 bp. In addition, a doublet was observed between 200 bp and 300 bp.

Using the lab-on-chip gel electrophoresis, three patterns were observed among the expected PCR-RFLP patterns for six Anguillid species (lanes 1-3, Fig. 3.3a). Lane 1 was similar to the *A. luzonensis* pattern, and the band sizes were 669, 367, 344, 171 and 110 bp. Lane 2 was similar to the *A. marmorata* pattern, and the band sizes were 837, 366, and 341 bp. Lane 3 had a similar pattern to *A bicolor pacifica* or *A. bicolor bicolor*, with band sizes of 824 and 681 bp. Lane 1 (Fig. 3.3b) also showed a similar pattern to *A. bicolor pacifica*, and the band sizes of 854, 278, and 223 bp confirmed that we were able to identify almost all *A. bicolor pacifica* (lane 1, Fig. 3.3b). There were 536 individuals identified by PCR-RFLP using *Msp* I. The 33 specimens distinguished as *A. bicolor pacifica* or *A. bicolor bicolor* by *Msp* I (lane 1; Fig. 3.3) were confirmed by *Dde* I as *A. bicolor pacifica* (lane 1, Fig. 3.3b). There were 16 specimens showing one of the unknown RFLP patterns similar to those in lanes 4-8 (Fig. 3.3a). No expected patterns were observed for *A. celebesensis* and *A. japonica*.

The phylogenetic tree (Fig. 3.4) with *Stemonidium hypomelas* as an outgroup showed that DNA sequences with expected and unknown RFLP patterns were grouped under its specific reference sequences. Individuals with an expected RFLP pattern similar to *A. luzonensis* (lane 1, Fig. 3.3a; LC588366-LC588367), the unknown pattern in lane 4 (Fig. 3.3a; LC588368), and a directly sequenced pattern (LC588369) were grouped under the *A. luzonensis* clade (AB469437; Fig. 3.4). The individuals with expected RFLP similar to *A. marmorata* (lane 2, Fig. 3.3a; LC588356-LC588358, LC588363- LC588364), unknown (lanes 5-6, Fig. 3.3a; LC588359-LC588362), and another individual directly sequenced pattern (LC588365) were grouped with the *A. marmorata* reference sequence (AP007242). Furthermore, individuals with a pattern similar to *A. bicolor pacifica* (lane 1, Fig. 3.3b; LC588370-LC588371) and unknown (lane 7, Fig. 3.3a; LC588373) were grouped under the clade of *A. bicolor pacifica* (AP007237). One of the sequenced

unknowns (lane 8, Fig. 3.3a; LC588374) belonged to the clade of *A. celebesensis* (AP007239).

3.3.2 Species composition

Anguilla luzonensis was found to recruit in the Lagonoy Gulf, mainly in the Comun and Lagonoy rivers, in addition to *A. marmorata* and *A. bicolor pacifica* (Fig. 3.5). Further, *A. luzonensis* was the second most abundant species in the Comun (9.5%) and Lagonoy (22.4 %) rivers, next to *A. marmorata. Anguilla marmorata* dominantly occur in the Comun (81.8%), Lagonoy (71.1%), and Bato (98.0%) rivers, while *Anguilla bicolor pacifica* was the third most abundant species in the Comun (7.7%) and Lagonoy (6.5%) rivers. In the Bato river, *A. luzonensis* and *A. bicolor pacifica* (1.0%) recruited at a considerably low percentage. In addition, a rare occurrence of *A. celebesensis* (0.9%) was observed only in the Comun river.

3.3.3 Population structure of A. luzonensis

When examining the genetic variability among the 663 nucleotide sequence sites obtained from 72 individuals, a total of 24 variable positions and 20 haplotypes were observed. The pairwise comparison based on the partial COI sequences of *A. luzonensis* from Comun and Lagonoy rivers showed low genetic diversity with an FST value of 0.00825, which was not significantly different ($p>0.05$) (Table 3.1).

The phylogenetic tree with *A. japonica* as an outgroup (Fig. 3.6) showed that almost all *A. luzonensis* individuals from Comun and Lagonoy and one individual from the Bato river formed a monophyly in one clade (B) with one individual diverged first (A) but node is only supported by 54% bootstrap value which is weak. In addition, the

two reference sequences downloaded from NCBI, which were found in the Cagayan river (*) also belonged to this large clade (B).

3.4 Discussion

3.4.1 Species identification

The simulation of the *Msp* I restriction enzyme digestion (Fig. 3.1) could distinguish *A. marmorata* (lane C), *A. japonica* (lane D), *A. luzonensis* (lane E), and *A. celebesensis* (lane F). Although *Msp* I cannot distinguish *A. bicolor pacifica* (lane A, Fig. 3.1) and *A. bicolor bicolor* (lane B, Fig. 3.1), *Dde* I could be used to distinguish *A. bicolor pacifica* from *A. bicolor bicolor*. In addition, the presence of the latter species in the Lagonoy Gulf may not be possible as it has previously been reported to occur only along the coasts of the Indian Ocean (Minegishi et al. 2012). Also, Shirotori et al. (2016) stated that the occurrence of *A. bicolor bicolor* reported in the Cagayan waters (Jamandre et al., 2007) was unconvincing due to insufficient method employed in distinguishing *A. bicolor pacifica* and *A. bicolor bicolor*; and one of their previous study (Aoyama et. al., 2015) in the same area only detected *A. bicolor pacifica*. Therefore, we judged that *Msp* I can be used for species identification of Anguillid species *A. marmorata*, *A. japonica*, *A. luzonensis*, and *A. celebesensis* (lanes C-F, respectively, Fig. 3.1). The use of *Dde* I in addition to *Msp* I could be used to distinguish *A. bicolor pacifica* from *A. bicolor bicolor* (Fig. 3.2; Fig. 3.3b).

The gel electrophoretic patterns showed that lane 5 (*Msp* I, Fig. 3.2), lane 3 (*Dde* I, Fig.3.2); lanes 1-4 and 8 (*Msp* I, Fig. 3.2), lanes 1-2 and 4-8 (*Dde* I, Fig.3.2); and lanes 6-7 (*Msp* I, Fig. 3.2) were similar to the simulated patterns of *A. bicolor pacifica* (lane A, Fig. 3.1), *A. marmorata* (lane C, Fig. 3.1), and *A. luzonensis* (lane E, Fig. 3.1), respectively. More than 200 bp bands were observed (Fig. 3.2) as predicted by the

simulated patterns (Fig. 3.1). However, the bands below 200 bp were not clearly observed (Fig. 3.2), although the simulation predicted that the digested bands between 100-900 bp could be detected (Fig. 3.1). In the *Msp* I digestion, a doublet between 700-900 bp was observed for *A. bicolor pacifica* (lane 5, Fig. 3.2) instead of the simulated two separate bands (lane A, Fig. 3.1). The thick band (lanes 1-4, 6-8, Fig. 3.2) between 300-400 bp with an intensity similar to the largest band may indicate that these were the doublet (340 bp, 370 bp) predicted by simulation for *A. marmorata* (lane C, Fig. 3.1) and *A. luzonensis* (lane E, Fig. 3.1). For *Dde* I digestion, lane 3 (Fig. 3.2) had a doublet between 200 bp and 300 bp instead of the separated bands in simulated pattern of *A. bicolor pacifica* (lane A; Fig 3.1). The PCR-RFLP patterns showed that the doublet of *A. bicolor pacifica* (lane 5) and the largest bands of *A. marmorata* (lanes 1-4 and 8) and *A. luzonensis* (lanes 6-7) for *Msp* I digestion can be used to distinguish these species (Fig. 3.2). *Anguilla celebesensis* showed no similar RFLP pattern as that of the simulated one (lane F, Fig. 3.1).

Anguilla marmorata, *A. luzonensis*, *A. bicolor pacifica* and *A. celebesensis* were confirmed by molecular analysis. The lab-on-chip gel electrophoresis showed that lanes 6 had a largest band of more than 800 bp, similar to the largest band for both *A. marmorata* (lane 2) and *A. bicolor pacifica* or *A. bicolor bicolor* (lane 3, Fig. 3.3a), hence, could not be identified. Doublets between 300-400 bp observed in lane 5 were similar to the doublets of *A. luzonensis* (lane 1) and *A. marmorata* (lane 2, Fig. 3.3a). However, the sequences of *A. marmorata* individuals with expected and unknown patterns that were grouped into one clade were the same species (100% bootstrap value).

The unknown RFLP patterns showed that lane 4 (Fig. 3.3a) had a largest band of around 671 bp, which was similar to a band detected in *A. luzonensis* (lane 1, Fig. 3.3a). *A. luzonensis* individuals showing expected and unknown patterns were grouped in one distinct clade, implying that these are the same species supported by 100% bootstrap value.

In the case of *A. bicolor pacifica*, the use of *Dde* I in addition to *Msp* I was able to confirm that the 33 individuals identified as *A. bicolor pacifica* or *A. bicolor bicolor* were *A. bicolor pacifica* individuals, and not *A. bicolor bicolor* (Fig. 3.3b). The largest band of lane 7 (more than 800 bp) is similar to the largest band for both *A. marmorata* (lane 2) and *A. bicolor pacifica* or *A. bicolor bicolor* (lane 3) (Fig. 3.3a), however, the DNA sequence was grouped with the specimens having expected RFLP patterns and reference sequence indicated that these are identical species. It may also be noted that, all detected bands of the *Msp* I digests of the three species the and the *Dde* I digest of *A. bicolor pacifica* were ranged within ±10% band sizing accuracy of the expected band sizes. Almost all individuals (536) were identified by PCR-RFLP using *Msp* I. Although we were not able to observe the expected pattern for *A. celebesensis*, we found an unknown individual (lane 8, Fig. 3.3a; LC588374) was *A. celebesensis*, since it was under the clade of its reference sequence (AP007239; Fig. 3.4).

PCR-RFLP using 16S rRNA have been reported appropriate for identification of the genus *Anguilla* (Aoyama et al., 2000; Watanabe et al., 2003; Watanabe et al., 2005) before *A. luzonensis* was discovered as a new species in 2009 (Watanabe et al., 2009). No available study used PCR-RFLP to identify *A. luzonensis*, hence, we simulated the restriction pattern for COI gene, in addition, to 16S rRNA for six target species and found that COI gene digested by *Msp* I and *Dde* I produced distinctive RFLP pattern for *A. luzonensis* and all the other five species. The combination of PCR-RFLP for COI gene by *Msp* I and *Dde* I and for unknown patterns using DNA sequencing analysis, finally, we were able to completely identify four Anguillid species in Lagonoy Gulf.

3.4.2 Species composition

Anguilla marmorata, *A. luzonensis*, *A. bicolor pacifica*, and *A. celebesensis* were found to recruit in the tributaries along Lagonoy Gulf. Among these species, *A. marmorata* was found to be dominant in all the rivers. A decrease on the percentage of recruitment of *A. marmorata* in Comun, Lagonoy and Bato rivers (87.5%, 82.3%, and 99.7% respectively) reported by morphology (Nieves et al., 2021) was due to the occurrence of *A. luzonensis* (9.5%, 22.4%, and 1%, respectively). Similarly, *A. marmorata* have dominantly recruited in Cagayan (Aoyama et al., 2015) and Mindanao (Shirotori et al., 2016). This means that *A. marmorata* is widely distributed in the Philippines.

It is quite interesting to note that *A. luzonensis* was found to be the 2nd most abundant species in the Comun and Lagonoy rivers. *Anguilla luzonensis* abundance and its stable recruitment for two periods of study was reported in Cagayan (Aoyama et al., 2015) and its rare occurrence in Mindanao (Shirotori et al., 2016). Our study and the references indicated the abundance of *A. luzonensis* in the northern and eastern part of the Philippines and its rarity in the south.

Anguilla bicolor pacifica was the third frequent species to occur only in the Comun and Lagonoy rivers. The molecular analysis found lower than 10% recruitment in Comun (7.7%) and Lagonoy (6.5%), which is less than the occurrence reported by morphological identification (12.4% and 17.5%, respectively) (Nieves et al., 2021) since we found that some *A. luzonensis* individuals were misidentified as *A. bicolor pacifica* by morphology. In Cagayan, an increase in the annual percentage of recruitment during two periods of their study was observed for *A. bicolor pacifica* (Aoyama et al., 2015). *Anguilla bicolor pacifica* was the 2nd most abundant species occurred in Mindanao (Shirotori et al., 2016). Based on our results and other studies, among A. bicolor subspecies, *A. bicolor*

pacifica is abundant in the whole Philippines. The decrease in the percent composition reported by morphology for *A. marmorata* and *A. bicolor pacifica* was due to the occurrence of *A. luzonensis* in Lagonoy Gulf.

In the Bato river, the *A. luzonensis* and *A. bicolor pacifica* species with low percentage occurrence may recruit during months when no samples were collected, since these were found to recruit in the Comun and Lagonoy rivers. *A. celebesensis* was rarely observed in the Comun river only, which is similar in Cagayan wherein this species was extremely rare (Aoyama et al., 2015). The use of morphology specific to pigmentation patterns was not able to distinguish *A. luzonensis* from *A. marmorata* (Nieves et al., 2021). Pigmentation patterns specific to *A. luzonensis*, A marmorata, and *A. celebesensis* are not yet established (Yoshinaga et al., 2014). The molecular analysis we carried out affirmed that pigmentation pattern alone is not enough to distinguish *A. luzonensis* among the pigmented eels (Leander et al., 2012). The species composition based on morphology can be confirmed and revised by molecular techniques. In addition, we were also able to confirm that no *A. bicolor bicolor* and only *A. bicolor pacifica* recruit in the Lagonoy Gulf. Hence, molecular analysis has provided a more precise estimate of Anguillid species composition in the tributaries along Lagonoy Gulf.

3.4.3 Population structure of A. luzonensis

Anguilla luzonensis was mostly found in two rivers, Comun and Lagonoy, along the Lagonoy Gulf. Individuals of *A. luzonensis* recruited in Comun and Lagonoy seem to have genetically variable sites and haplotypes. In addition, *A. luzonensis* between these two rivers were found to have low genetic diversity, which was not statistically significant (Table 3.1), possibly indicating a panmictic population. The dispersed *A. luzonensis* individuals from the Comun and Lagonoy rivers in clade B (Fig. 3.6), including the two

DNA sequences downloaded from NCBI, which were collected from Cagayan, could imply that these individuals share similar genetic materials. *Anguilla luzonensis* and *A. japonica* were presumed to have similar spawning area (Han et al., 2016; Kuroki et al., 2012), experience similar oceanographic features (Han et al., 2016), though distribution are different, wherein the latter was reported to have a single panmictic population in the East Asia (Han et al., 2010). Although our study was only in Lagonoy Gulf, *A. luzonensis* might have a similar case with *A. japonica* having panmictic population. Since this is the first investigation of the population structure of *A. luzonensis* recruited in tributaries along the Lagonoy Gulf and being the second abundant species that may have high economic importance, this study could indicate important implications for resource management of this species. Furthermore, genetic differences and population structure of *A. luzonensis* from Luzon, Mindanao, Philippines, Taiwan, and Okinawa, Ryuku archipelago may be compared and studied in the future.

This study was able to clarify the species composition of pigmented Anguillids recruited in the tributaries along Lagonoy Gulf. The PCR-RFLP analysis by *Msp* I and/or *Dde* I followed by DNA sequencing only, when necessary, found *A. marmorata*, *A. bicolor pacifica*, *A. luzonensis*, and *A. celebesensis*. The new species *A. luzonensis* was confirmed to exist and was the second most-abundant species to occur. Although genetic variability was found in *A. luzonensis* individuals, genetic diversity was very low and not significantly different, which was inferred from the partial COI gene fragment. It may be noted that our current study was not able to find enough *A. luzonensis* individuals from Bato river but highly possible to occur during months with no samples were collected; therefore, this study will be further continued. With *A. luzonensis* classified as a vulnerable species and occurrence of the high-value species *A. bicolor pacifica*, this study provides information that will be of help to effective management and utilization of

freshwater eels. The dominance of the low-value species, *A. marmorata*, also indicated its potential for product development to improve its marketability in the region. Focusing on the effective processing methods of *A. marmorata*, may entice aquaculture owners to grow their glass eels until the adult stage that may lead to new products, and create new markets and avenues for the fishermen to improve their livelihood.

Table 3.1 Genetic diversity based on the partial COI (663 bp) gene of *A. luzonensis* individuals collected from Comun and Lagonoy rivers.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage variation	Fixation index (F_{ST})
Among populations	1	1.234	0.00823	0.82	
Within populations	70	69.252	0.98932	99.18	0.00825

p-value>0.05; 10,230 permutations

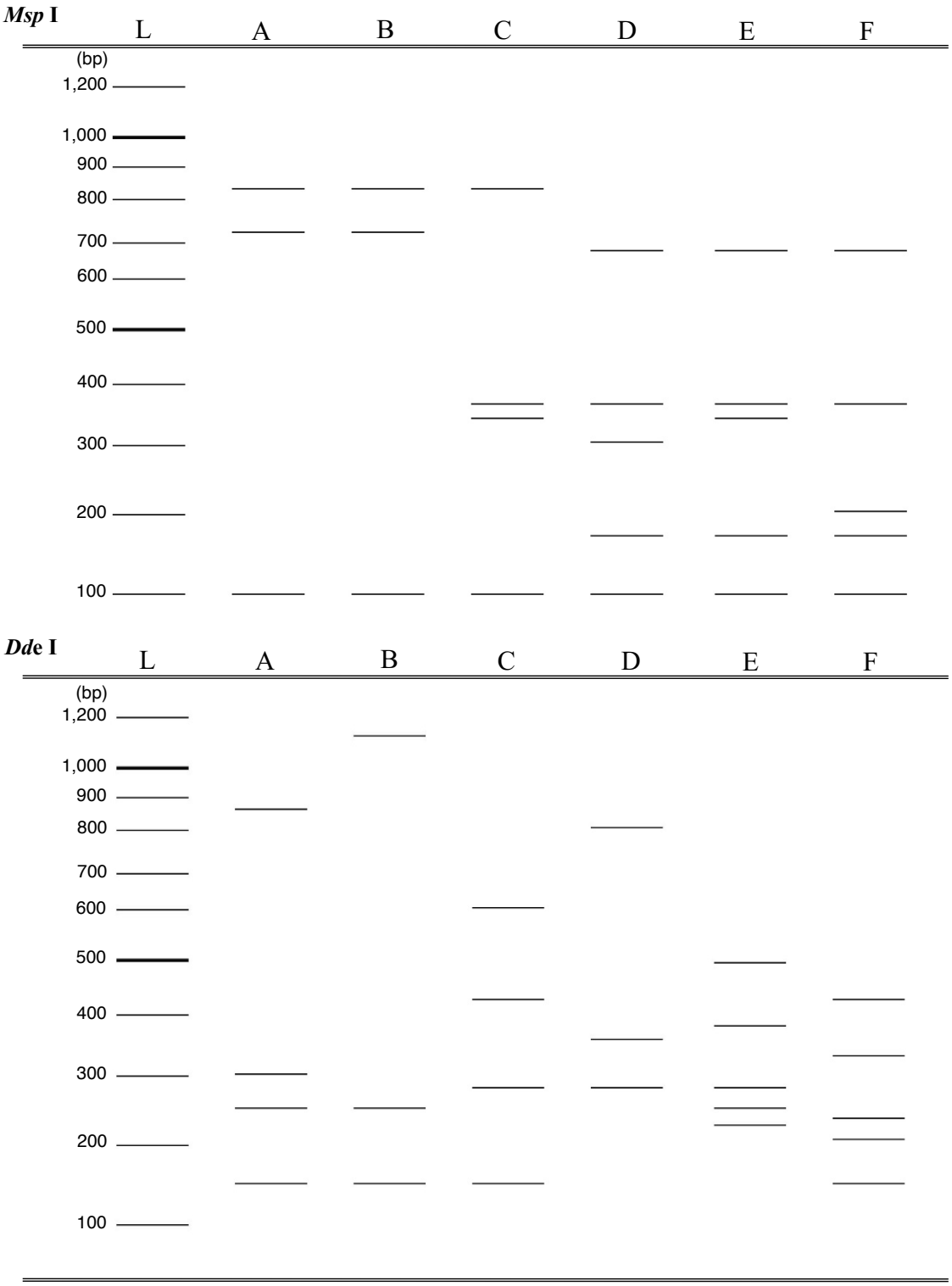


Fig. 3.1 Simulated PCR-RFLP patterns of the 1,635 bp COI target fragment gene with location 5,503-7,138 bp in the mitochondrial genome.
Restriction enzyme: *Msp* I, *Dde* I; Lane L, molecular marker; (A) *A. bicolor pacifica*; (B) *A. bicolor bicolor*; (C) *A. marmorata*; (D) *A. japonica*; (E) *A. luzonensis*; and (F) *A. celebesensis*. bp, base pairs.

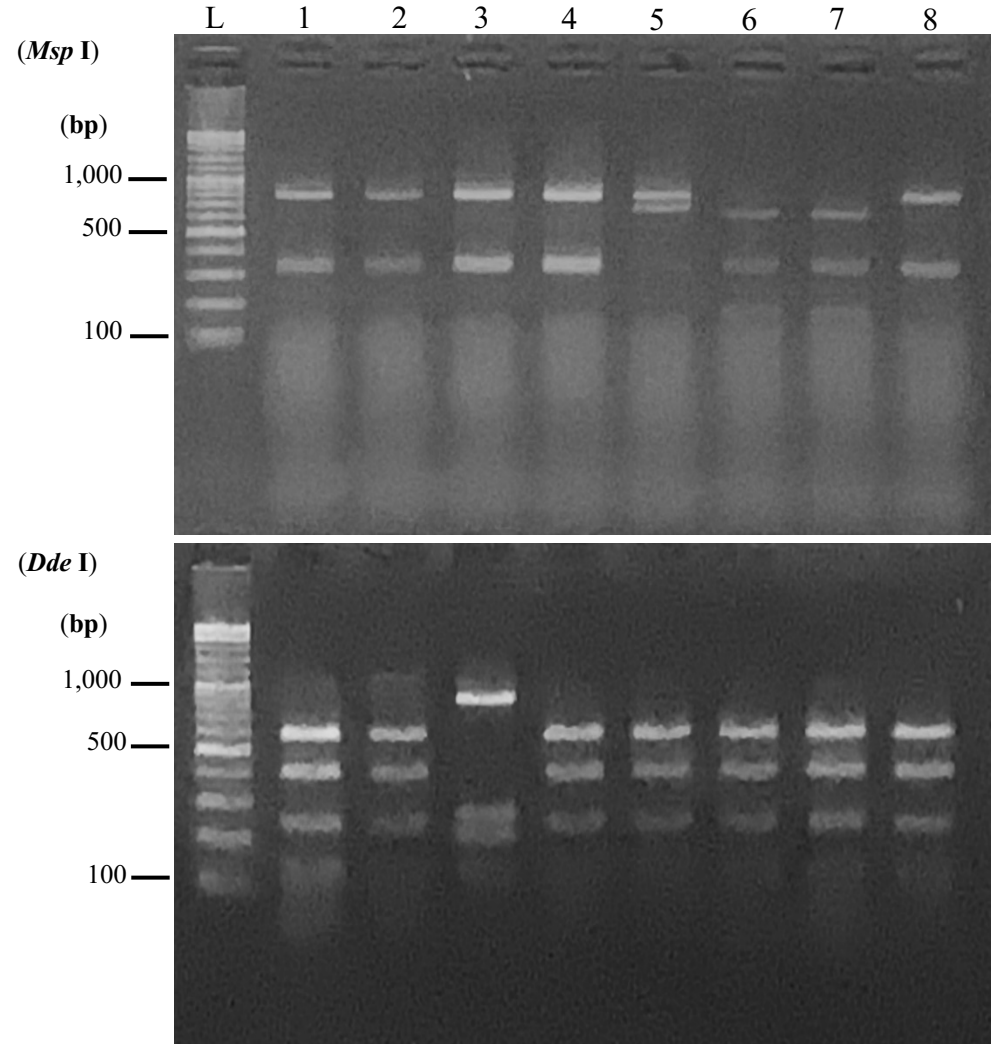


Fig. 3.2 Gel electrophoretic patterns of *A. marmorata*, *A. bicolor pacifica* and *A. luzonensis* after *Msp* I and *Dde* I digestion.
Lane L, ladder; Lanes 1-4, 8 for *A. marmorata*; lane 5, *A. bicolor pacifica*; lane 6-7, *A. luzonensis* (*Msp* I, upper photo); lanes 1-2, 4-8, *A. marmorata*; lane 3, *A. bicolor pacifica* (*Dde* I, lower photo); bp, base pairs

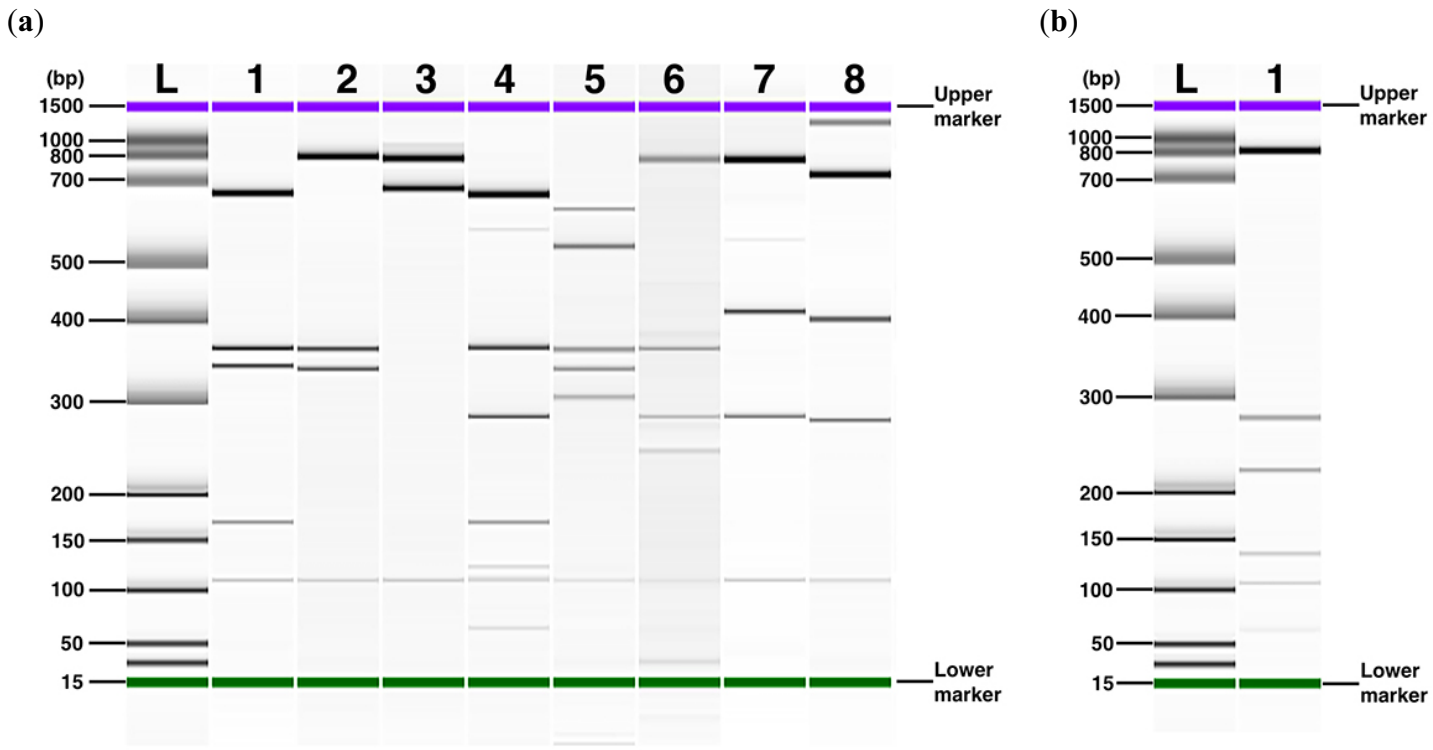


Fig. 3.3 Restriction digestion by *Msp* I for *A. luzonensis*, *A. marmorata*, *A. bicolor pacifica* or *A. bicolor bicolor* and unknown PCR-RFLP patterns of *A. luzonensis*, *A. marmorata*, *A. bicolor pacifica*, and *A. celebesensis*; and *Dde* I for *A. bicolor pacifica*. Restriction enzymes (**a**) *Msp* I and (**b**) *Dde* I; L, Ladder; (**a**) lane 1, *A. luzonensis*; 2, *A. marmorata*; 3, *A. bicolor pacifica* (expected). Lane 4, *A. luzonensis*; 5-6, *A. marmorata*; 7, *A. bicolor pacifica*; 8, *A. celebesensis*; unknowns confirmed by DNA sequencing analysis; and **(b)** lane 1, *A. bicolor pacifica*; bp, base pairs

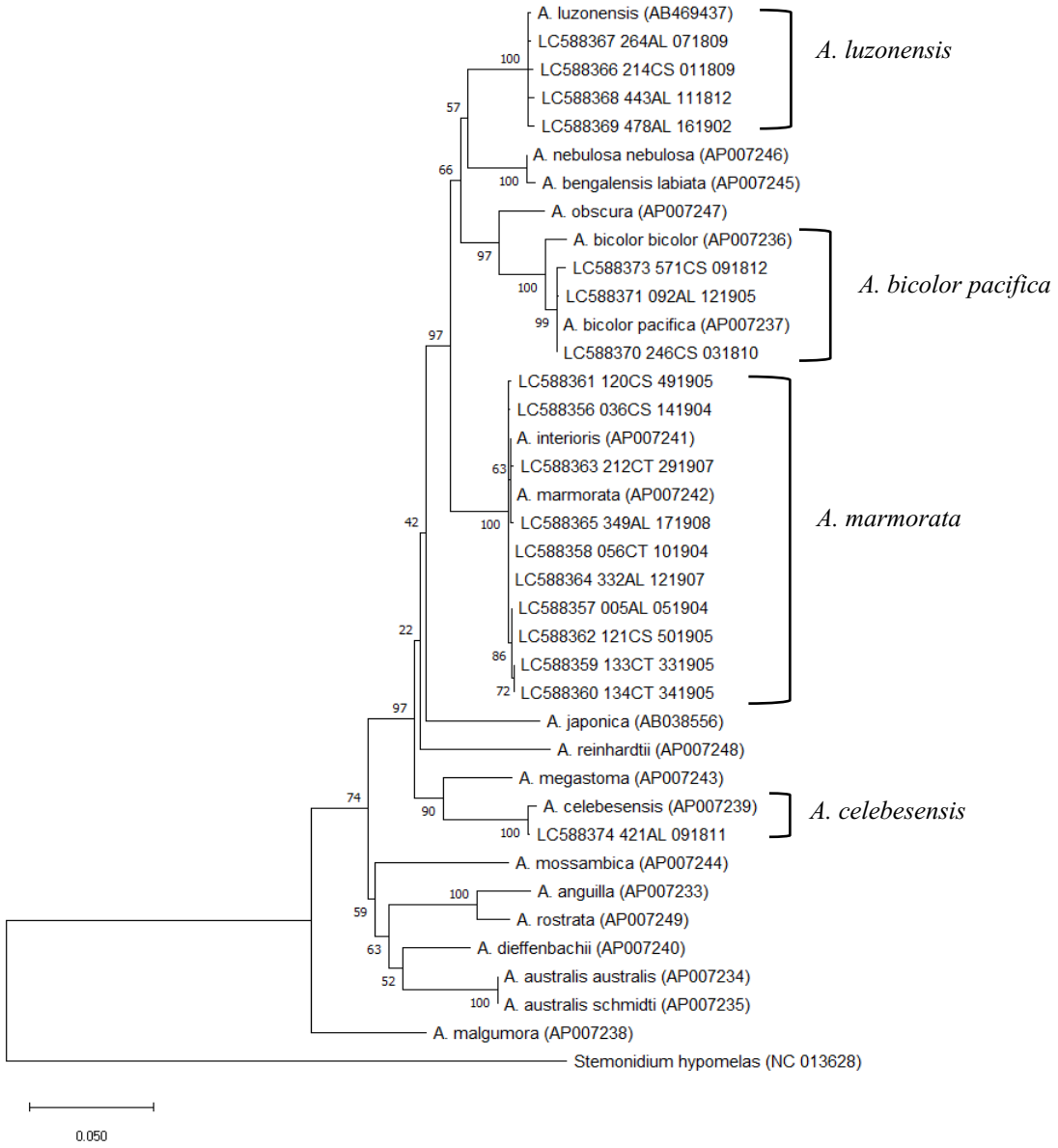


Fig. 3.4 Maximum likelihood tree of the DNA sequences of specimens showing typical and unknown RFLP patterns.

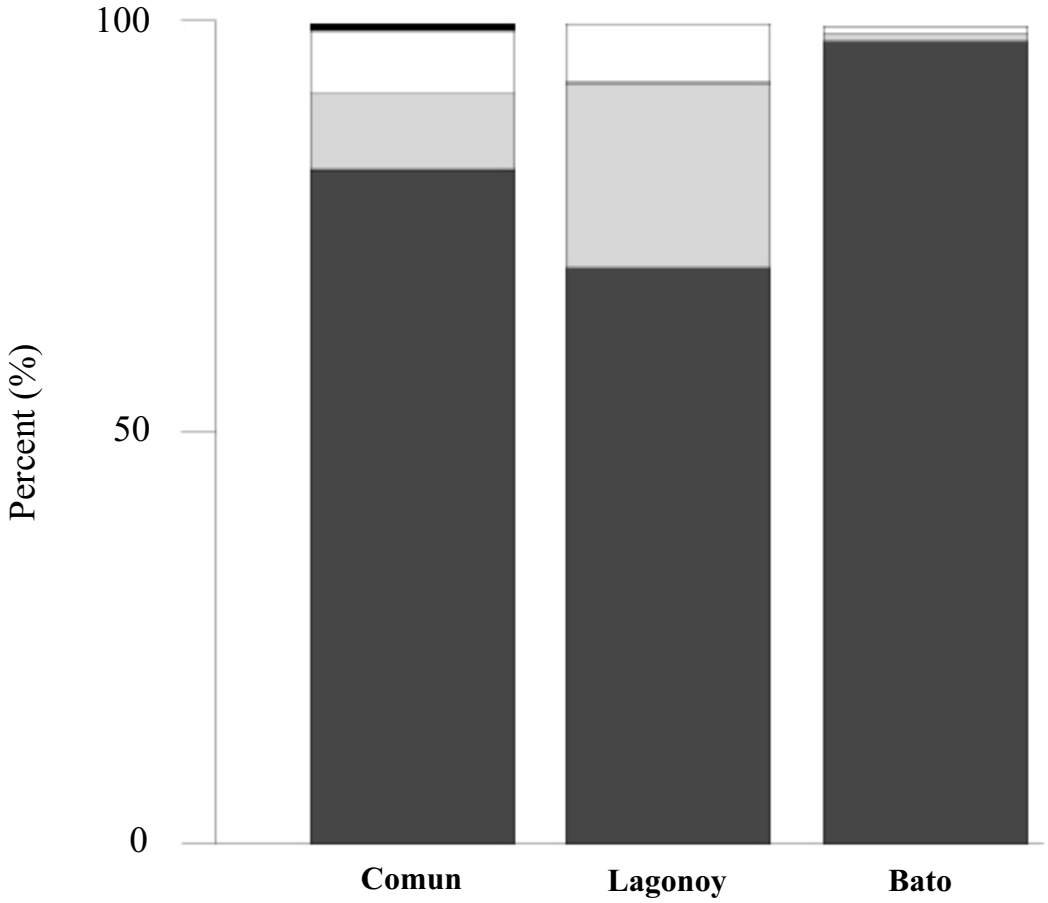


Fig. 3.5 Percent (%) composition of freshwater eels recruiting in each of the rivers. ■, *A. marmorata*; □, *A. luzonensis*; □, *A. bicolor pacifica*; ■, *A. celebesensis*

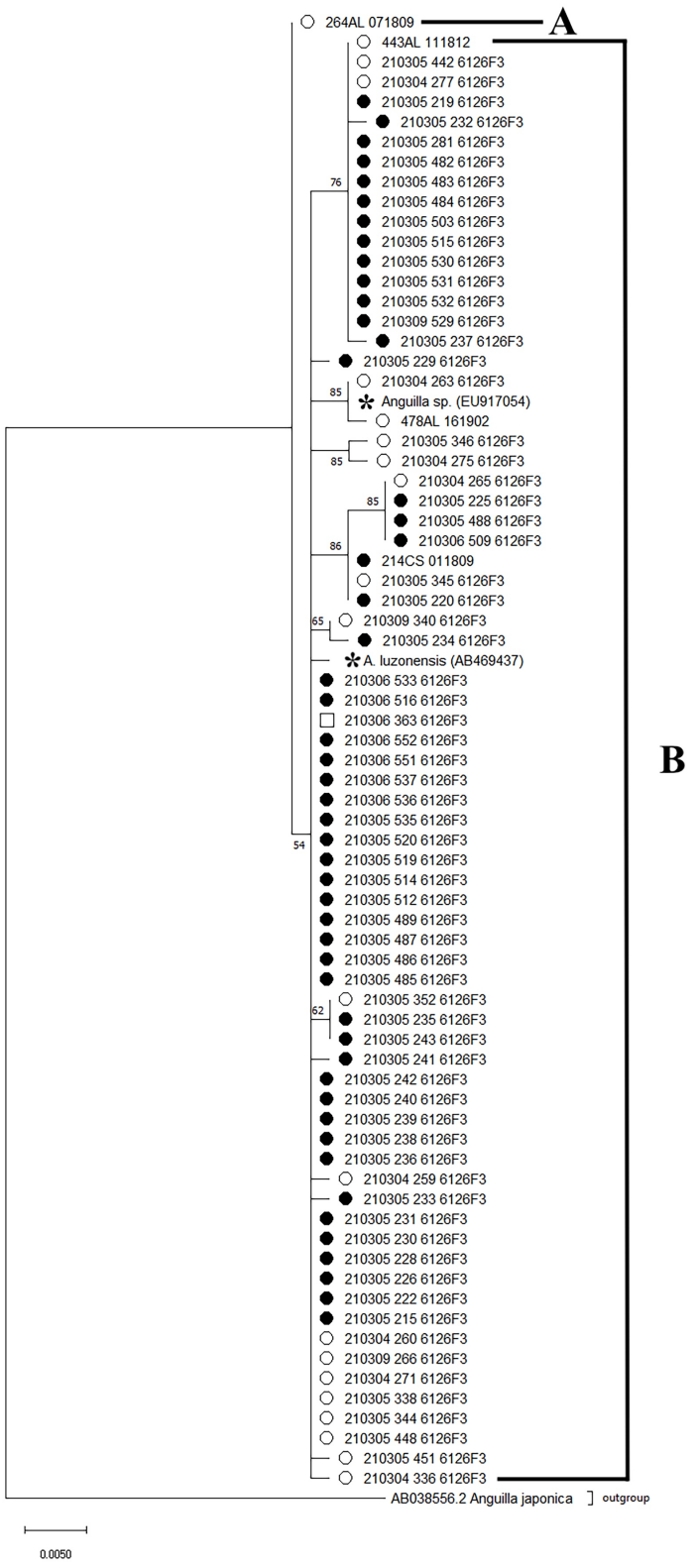


Fig. 3.6 Maximum likelihood tree showing phylogenetic relationships between *A. luzonensis* collected mainly from Comun (o) and Lagonoy (●) and one individual from Bato (▫) river. Reference sequences (AB469437, EU917054) of *A. luzonensis* from Cagayan (*) downloaded from NCBI was also included.

Chapter 4 Comparison of Morphological and Molecular Identification

Abstract

Pigmented glass eels recruited in Lagonoy Gulf were initially grouped as *A. marmorata* and *A. bicolor pacifica* due to difficulty in distinction by pigmentation patterns (Chapter 2). Confirmation by molecular analysis revealed the existence of *A. luzonensis* and *A. celebesensis* in addition to *A. marmorata* and *A. bicolor pacifica*. Hence this chapter has clarified the pigmentation pattern useful for identifying *A. marmorata*, *A. luzonensis*, and confirm for *A. bicolor pacifica*. Among the morphologically identified *A. marmorata*, 81.7% were correctly identified, whereas part of it were confirmed as *A. bicolor pacifica* (4.4%) and *A. luzonensis* (13.9%). In the case of *A. bicolor pacifica*, only 16.7% among all morphologically identified were correct. It turned out that there were several *A. marmorata* (70.8%), *A. luzonensis* (9.7%), and *A. celebesensis* (2.8%) specimens that were misidentified as *A. bicolor pacifica* by pigmentation pattern. Re-observation of the photos of 140 individuals, distinction of species can only be between groups of *A. bicolor pacifica* and *A celebesensis* having pigmentation pattern reaching until the tip of caudal fin (pattern a); and *A. marmorata* and *A. luzonensis* with pigmentation patterns on the tail not reaching the caudal fin tip (patterns b-g). However, inconsistencies on the initial observation of the pigmentation patterns and re-observation caudal pigmentation photos especially for *A. bicolor pacifica* were revealed. Almost 80.6% of the morphologically identified *A. bicolor pacifica* were misidentified as *A. marmorata* and *A. luzonensis*, although the re-observation showed that distinction between *A. bicolor pacifica* from *A. marmorata* and *A. luzonensis* was possible. Nevertheless, the use of pigmentation pattern alone for species identification is not enough, hence, combination with molecular analysis is recommended. Moreover, the use

of molecular analysis is better for precise identification of Anguillids recruited in Lagonoy Gulf.

4.1 Introduction

Anguilla marmorata, *Anguilla luzonensis*, and *Anguilla bicolor pacifica* were the main pigmented glass eels reported to occur in the tributaries along Lagonoy Gulf by molecular analysis (Chapter 3). The previous study using pigmentation pattern has only grouped individuals exhibiting pigments in the tail and caudal fin as *A. marmorata* and *A. bicolor pacifica* (Chapter 2). The pigmentation pattern seems to easily distinguish *A. bicolor pacifica* from other pigmented glass eels recruited in Lagonoy Gulf (Chapter 2). On the other hand, it was not easy to distinguish *A. luzonensis* from *A. marmorata* due to similarity in the pigmentation patterns (Chapter 2). Hence, all specimens with unclear pigmentation patterns were grouped as *A. marmorata*. However, confirmation of the species identification by molecular analysis revealed the occurrence of *A. luzonensis* in addition to *A. marmorata* and *A. bicolor pacifica* (Chapter 3). We found that the identification of Anguillid glass eels using pigmentation patterns was not enough to be used for identification of Anguillids recruiting in Lagonoy Gulf.

Morphological identification is a traditional method of identification of Anguillid species (Tabeta et al., 1976a; Tabeta et al., 1976; Watanabe et al., 2004). At the glass eel stage, the sectional counts of vertebrae and caudal pigmentation pattern, which facilitated the identification by observation under a stereomicroscope, were used for identification (Tabeta et al., 1976; Tabeta & Mochioka, 2003). *Anguilla japonica* lacks caudal pigmentation and *A. bicolor pacifica* with small spots that appear first on the caudal fin and developed anteriorly during growth (Tabeta et al., 1976; Tabeta & Mochioka, 2003). *Anguilla bicolor pacifica* can be identified by the pigmentation pattern on caudal fin reaching its tip (Leander et al., 2012; Han et al., 2012). Morphological identification using pigmentation pattern have long been practiced by fishers in the field setting, however, this

had been considered a problem by fishers due to the difficulty in distinction of Anguillid species at the glass eel stage (SEAFDEC, 2019; personal communication).

Studies have compared morphological and molecular identification of Anguillids and concluded that molecular analysis was found more suitable for species identification (Sugeha et al., 2008; Watanabe et al., 2005). Several studies combined both morphological and molecular tools for the identification of glass eels. Specimens were initially separated based on the fin-type then the caudal pigmentation wherein, short-finned with pigmentation until the tip of the caudal fin is *A. bicolor pacifica* and pigment-absent specimens were *A. japonica* (Han et al., 2012; Leander et al, 2012). The use of the ano-dorsal and total length relationship expressed in percentage may identify *A. marmorata* with ano-dorsal length (ADL)/ total length (TL) of >13%, while other long-finned specimens with ADL/TL and <13% were found to be *A. marmorata*, *A. celebesensis*, and *A. luzonensis* by molecular analysis (Han et al., 2012; Leander et al., 2012). Succeeding studies have also employed a similar method in identification, with one study having slight modifications (Yoshinaga et al., 2014; Aoyama et al., 2015; Shirotori et al., 2016). *Anguilla japonica* and *A. bicolor pacifica* were reported to be identified by morphology even without further molecular analysis (Han et al., 2016). On the other hand, *A. marmorata* and *A. luzonensis* cannot be distinguished using caudal pigmentation patterns (Leander et al., 2012; Yoshinaga et al., 2014). Nevertheless, representative samples of specimens identified by morphology still underwent molecular analysis to confirm precise species identification.

Therefore, this chapter has clarified the pigmentation pattern useful for identifying *A. marmorata*, *A. luzonensis* and confirm for *A. bicolor pacifica*.

4.2 Materials and Methods

4.2.1 Glass eel samples

Glass eel caudal fin and photos of several individuals (n= 140) were observed again under a stereomicroscope (2x magnification) and then compared.

4.2.2 Molecular and morphological comparisons

The confirmed molecular identification of the specimens was compared with the morphological identification based on the pigmentation patterns. Percentage of the correct and misidentified individuals using the morphological identification was computed based on the molecular analysis result and is expressed in percentage (%).

4.3 Results

4.3.1 Pigmentation patterns

Fig. 4.1 shows the photos of the pigmentation pattern on the tail of glass eel specimens identified for *A. marmorata* (Fig. 4.1b-h) and *A. bicolor pacifica* (Fig. 4.1a). Fig. 4.1a shows dense pigment patches on the caudal fin reaching the tip. Also, less dense pigmentation on the tail was observed. These are typical characters of *A. bicolor pacifica*. On the contrary, Fig. 4.1b shows uniformly distributed small pigment patches on the tail. Whereas Fig. 4.1c shows gradation in the pigment patches on the tail. In Fig. 4.1c to 4.1e, three types of pigment patches with a gradation from the anterior to the posterior part of the tail can be seen. A decreasing trend in the density of the anterior and posterior parts of the tail is also observed. In Fig. 4.1f, background coloration on the tail is prominently seen. Also, the gradation of pigment patches can be observed on the tail. Fig. 4.1g also shows background coloration on the tail. However, the anterior part of the tail in Fig. 4.1g

has less dense background coloration compared to Fig. 4.1f. Also, almost no pigment patches are visible on the tail of Fig. 4.1g.

4.3.2 Molecular and morphological comparisons

Table 4.1 shows the proportion of the *Anguilla* species individuals identified by morphology and, subsequently, confirmed by molecular analysis. Of the 482 individuals identified morphologically as *A. marmorata*, 81.7% were correctly identified, whereas 4.4% *A. bicolor pacifica* and 13.9% *A. luzonensis* specimens were misidentified as *A. marmorata*. In the case of *A. bicolor pacifica*, only 16.7% of the 72 individuals were correctly identified by the pigmentation pattern. It turned out that there were several *A. marmorata* (70.8%), *A. luzonensis* (9.7%), and *A. celebesensis* (2.8%) specimens that were misidentified as *A. bicolor pacifica*.

Based on the photos observed for 140 individuals (Table 4.2), pattern a, which is a typical character of *A. bicolor pacifica*, was confirmed to be correct (88.9%). However, 11.1% of the morphologically identified *A. bicolor pacifica* were confirmed to be *A. celebesensis*. On the contrary, patterns c (100%), d (95.7%), f (100%), and g (83.3%) were confirmed to be exhibited mainly by *A. marmorata*. Whereas patterns b (86.4%) and e (100%) were confirmed to mostly for *A. luzonensis*.

4.4 Discussion

4.4.1 Pigmentation patterns

Different pigmentation patterns for *A. marmorata* and one pattern for *A. bicolor pacifica* were observed and identified. The pigmentation pattern typical for *A. bicolor pacifica* was easily distinguished by the pattern on the tail reaching until the caudal fin (Fig. 4.1a). In the case of *A. marmorata*, different patterns were observed characterized

by the small patches which were uniformly distributed (Fig. 4.1b), having gradations that with a decreasing trend from the anterior to posterior part of tail (Fig. 4.1c-e) and background coloration (Fig. 4.1f-g) on the tail. A distinct pigmentation pattern reaching until the caudal fin tip observed for *A. bicolor pacifica* can easiliy distinguish it from a group of pigmented glass eels unlike *A. marmorata* having different patterns on the tail not reaching the tip of the caudal fin.

4.4.2 Molecular and morphological comparisons

Among all the *A. marmorata* morphologically identified, a high percentage (81.7%) were confirmed to be correctly identified by the pigmentation pattern. Misidentification of *A. bicolor pacifica* and *A. luzonensis* by pigmentation patterns was confirmed (4.4% and 13.9%, respectively). In the case of the morphologically identified *A. bicolor pacifica*, only low percentage (16.7%) were confirmed correctly identified whereas mostly were misidentified as *A. marmorata* (70.8%), *A. luzonensis* (9.7%), and two individuals of *A. celebesensis*.

Observing the pigmentation pattern again, pattern a (Fig. 4.1a) with dense pigmentation on the tail reaching the caudal fin tip seems to be clearly distinct from the other pigmentation patterns (Fig. 4.1b-g). In contrast, patterns b-g were the pigmentation patterns not reaching the caudal fin tip. However, patterns b-e have pigment patches which were almost not visible in patterns f and g.

Both *A. bicolor pacifica* (8 individuals; Table 4.2) and *A. celebesensis* (1 individual; Table 4.2) exhibited pattern a (Fig. 4.1a). No individuals of *A. bicolor pacifica* and *A. celebesensis* were found having pigmentation patterns b-g (Table 4.2), hence, pattern a characterized by the dense pigmentation on the tail reaching the caudal fin tip is only for *A. bicolor pacifica* and *A. celebensensis* that can make distinction difficult.

Currently, no publication has described the pigmentation patterns of *A. celebesensis* and this study first described it however only one individual was observed, hence we cannot conclude on its distinction only by pigmentation. However, if in case there are many *A. celebesensis* individuals, pattern a cannot be used to identify *A. celebesensis* from *A. bicolor pacifica*.

Only *Anguilla marmorata* (66 individuals) and *A. luzonensis* (11 individuals) exhibited patterns c (Fig. 4.1c) and e (Fig. 4.1e), respectively. Two individuals of *A. marmorata* were observed to have pattern f (Fig. 4.1f) and none in *A. luzonensis*. *Anguilla marmorata* and *A. luzonensis* were both observed having patterns c, d and g. However, *A. marmorata* mostly exhibited pattern d and g (22 and 5 individuals respectively) and only one individual of *A. luzonensis* were observed to have this pigmentation patterns. While most of *A. luzonensis* (19 individuals) exhibited pattern b and three (3) individuals of *A. marmorata* also showed the same pigmentation pattern. Not all individuals showed a 100% like pattern c and e. Other patterns are comprised of mixed species of individuals of *A. marmorata* and *A. luzonensis*. However, distinction of *A. marmorata* and *A. luzonensis* individuals using pigmentation patterns on the species level is difficult. We can only separate the group of individuals as a group composed of *A. bicolor pacifica* and *A. celebesensis* (pattern a) from the group of individuals comprised of *A. marmorata* and *A. luzonensis* (patterns b-g).

Inconsistencies with the observation on pigmentation patterns (Chapter 2) and re-observation by photos again in this chapter were observed for *A. bicolor pacifica*. Among the 554 individuals, 72 individuals (13%) were distinguished by pigmentation pattern as *A. bicolor pacifica*. However, the 72 individuals were confirmed by molecular analysis to have *A. bicolor pacifica*, *A. marmorata*, and *A. celebesensis*. Molecular analysis showed that most of the *A. bicolor pacifica* were misidentified (83.3%). The re-

observation revealed that *A. bicolor pacifica* could be distinguished from *A. marmorata* and *A. luzonensis* but not with *A. celebesensis*. The pace of observation, the huge number of samples analyzed, and the relative abundance of the species collected in Lagonoy Gulf may have influenced these inconsistencies. In addition, a scattered pigment patches nearly reaching the caudal fin were observed that may have been perceived and characterized by the observer for *A. bicolor pacifica*. Hence the possibility of subjective judgment and the observer's experiences for morphological identification may also have influenced the discrepancies of the observations made.

Nevertheless, the use of pigmentation pattern alone is not enough; combining it with molecular analysis is necessary. Due to the urgency in distinction among Anguillid species caught by fishermen, pigmentation patterns are commonly used; however, with the similarities of pigmentation patterns, identification of the species had been a challenge. The precise distinction of Anguillids with pigmentation patterns on the tail and caudal fin i.e., *A. marmorata* and *A. luzonensis* is quite impossible just by using pigmentation patterns. Hence molecular analysis is better to be used for precise identification.

Table 4.1 Percentage (%) of Anguillid species correctly identified and misidentified using pigmentation pattern confirmed by molecular analysis.

	Molecular			
Morphology	*A. marmorata*	*A. bicolor pacifica*	*A. luzonensis*	*A. celebesensis*
A. marmorata n=482	81.7	4.4	13.9	0
A. bicolor pacifica n=72	70.8	16.7	9.7	2.8

Note: Data in the table are expressed in percentage (%); computed percentage in each of the column were based on the molecular analysis result.

Table 4.2 Confirmation of the morphological species identification by molecular analysis.

Morphology	**Molecular**			
Patterns	***A. bicolor pacifica***	***A. marmorata***	***A. luzonensis***	***A. celebesensis***
A	88.9 (8)	0	0	11.1 (1)
B	0	13.6 (3)	86.4 (19)	0
C	0	100 (66)	0	0
D	0	95.7 (22)	4.3 (1)	0
E	0	0	100 (11)	0
F	0	100 (2)	0	0
G	0	83.3 (5)	16.7 (1)	0

Note: Data in the table are expressed in percentage %; values in parenthesis ( ) are the number of individuals

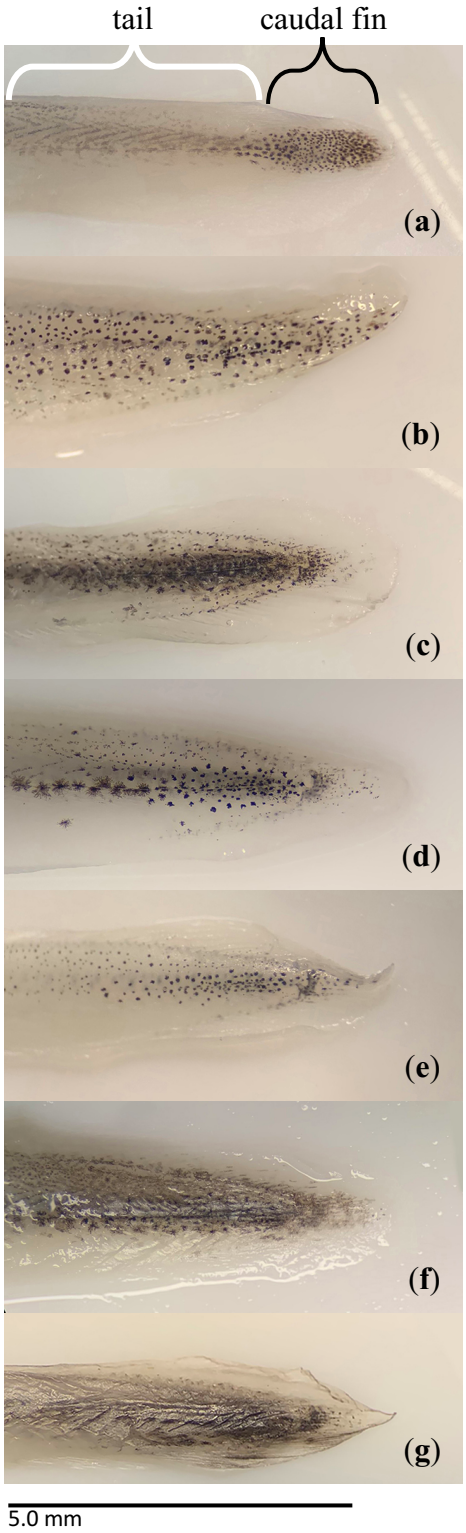


Fig. 4.1 Photo of the caudal pigmentation patterns exhibited by the glass eel specimens. White bracket indicates tail and black bracket is for caudal fin. Scale bar size 5.0 mm.

Chapter 5 Gelatin in the *Paksiw* Soup of *Anguilla marmorata* and *Anguilla bicolor pacifica*

Abstract

Anguilla marmorata, the most abundant species in the Philippines, has a high potential for food development due to comparable nutritional composition and superiority in terms of preference test to the high valued *A. bicolor pacifica*; and the possibility of high collagen content being the same genus with *A. japonica*. Anguillids cooked as *paksiw*, one of the popular cuisines in the Philippines, prepared by boiling fish, could produce gelatin, a taste enhancer. Therefore, this study investigated the contribution of the eel parts in the gelatin content of *paksiw* soup made from *A. marmorata* and *A. bicolor pacifica* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and determined the protein concentration by Coomassie Protein Assay. The SDS-PAGE pattern showed that gelatinous proteins observed within the vicinities of 116 kDa and 205 kDa were only found in the cooked *paksiw* soup of *A. marmorata* and *A. bicolor pacifica* and none in the uncooked ones. The non-gelatinous proteins less than 97.4 kDa were mostly observed in the uncooked *paksiw* soup. Gelatin is mostly derived from the skin as observed with the highest staining intensity among eel parts. Yet protein concentration determined by Coomassie protein assay showed higher values in muscles than the skin which is in contrast with the staining intensities observed in the SDS-PAGE analysis. However, the densitometric analysis conducted revealed higher protein concentration of the skin than the muscle which agrees with the SDS-PAGE observation. Based on the results of the SDS-PAGE analysis and the determined protein concentration, it may be deduced that gelatin content in the soup of *paksiw* made from *A. marmorata* is comparable with *A. bicolor pacifica*. Further analysis on the free and gelatin-bound hydroxyproline, estimation of the total gelatin content, taste active components and taste

enhancement effect of gelatin by preference test of the large-scale *paksiw* will be conducted in the future.

5.1 Introduction

Three species of freshwater eels were found to recruit in the tributaries along Lagonoy Gulf. *Anguilla marmorata* is the most abundant species, followed by *Anguilla luzonensis* and *Anguilla bicolor pacifica* (Chapter 3). *Anguilla marmorata* is of lower market value than *A. bicolor pacifica* (Crook 2014), although studies have shown that their nutritional composition is comparable (Ahn et al. 2015; Luo et al. 2015). In addition, the preference test of grilled *A. marmorata* and *A. bicolor pacifica* showed similarities in terms of taste (Ahn et al., 2015). However, *A. marmorata* was reported to have a slightly better evaluation in terms of chewiness and a higher rank in terms of the general acceptability than *A. bicolor pacifica* (Ahn et al., 2015). Therefore, market-driven evaluation of *A. marmorata* is lower than *A. bicolor pacifica* since *A. bicolor pacifica* is the species being preferred in the export market (Crook 2014; SEAFDEC 2019). *Kabayaki* is the most famous way of cooking freshwater eels such as *Anguilla japonica* and *A. bicolor pacifica* in Japan, Taiwan, China, and Korea (Dou, 2014; Kuroki et al., 2014; Lee, 2014; Tzeng, 2014); however, this dish is not known in the Philippines. Sour-stewed— *sinigang*, *paksiw*; steamed—*pinasingaw*, *halabo*s; *roasted— inihaw*; and boiled— *nilaga*, are native cuisines of the Philippines (Fernandez, 1988). Among these cuisines, *paksiw* is one of the famous in the country (Fernandez, 1988); hence we tried to utilize this cooking method *A. marmorata* and *A. bicolor pacifica*.

Paksiw is a native Filipino cuisine wherein fish, or meat, is stewed in vinegar which has evolved due to the need to preserve without refrigeration (Fernandez, 1988). *Paksiw* is characterized by the sour-salty and "*malinamnam*" taste (personal communication), wherein "*malinamnam*" could refer to the mouthfeel not only of the fish but also its soup. Although *paksiw* is known with different terms such as *pinaksiw* in Tagalog, *inun-unan* in Cebuano and Boholano; *paksi* in Kapampangan; *nilengla* in

Ilocono; *piyalam* in Ta'u-sug; and *inon-on* in the Bikol (Merano, n.d.-b, n.d.-a; Polistico, 2017; The Freeman, 2016), the cooking way is similar. Fish *paksiw* or locally known as *paksiw na isda*, is a recipe of any fish commonly being boiled and simmered with water, vinegar (between 1:0.5 and 1:2 proportions), and ginger (1-2 knob/thumb; 0.5-30 g) (Asian Food Network, 2020; Boquet, 2017; DOST-FNRI 2018; Fernandez, 1988; Merano, n.d.-b, n.d.-a; Nutriasia, 2021; Tiangson-Bayaga & Deveza, 2005; Yap et al., 2007). Aside from these ingredients, additional spices like garlic (4-5 cloves; 1-16 g), onion (1 small to medium size), salt (1-2 tsp), whole black peppers (½-1 tsp; 1-5 g; 5-10 pcs) and vegetables such as bitter gourd (1-2 pcs or ½ cup sliced into pieces one small to medium size or ½-2 ½ cups sliced into pieces) and eggplant (1 pc or ½-2 1/8 cups sliced into pieces) can also be used (Asian Food network 2020; Boquet 2017; DOST-FNRI, 2018; Merano, n.d.-b, n.d.-a; Nutriasia, n.d.; Tiangson-Bayaga & Deveza, 2005; Yap et al. 2007). This recipe can be prepared with Milkfish (*bangus*), Gobies (*biya*), Black Finned Mullet (*talilong*), Long-Finned Mullet (*banak*), Japanese Bigeye (*buwan-buwan* or *kwaw*), Ten Pounder (*bidbid*), Spadefish (*kitang*), Anchovy (*dilis* or *bolinao*); Blue Mackerel Scad (*galunggong*), Tuna (*tulingan*), Slipmouth fish (*sapsap*), or Tilapia (Asian Food Network, 2020; Boquet, 2017; DOST-FNRI 2018; Fernandez, 1988; Merano, n.d.-b, n.d.-a; Nutriasia, 2021; Tiangson-Bayaga & Deveza, 2005; Yap et al., 2007; personal communication). The *paksiw* recipe has been standardized using milkfish by Tiangson-Bayaga and Deveza (2005) based on a survey. Although not famous for consumption in this generation, freshwater eels (locally known as *igat* in the country or *kasili* in Bikol) such as *A. marmorata* or *A. bicolor pacifica* have been cooked as *paksiw* and served only during special occasions (Chapter 2). Fishes are generally high in proteins, excluding water, and low in carbohydrates (Belitz et al., 2009; Tahergorabi et al., 2011; Venugopal & Shahidi, 1996). During the boiling process in *paksiw*, most of the fish proteins are

precipitated and one of the abundant proteins, collagen (in the form of gelatin), is solubilized (Qixing et al., 2014; Sikorski & Borderias, 1994; Tornberg, 2005; Zhang et al., 2013). Gelatin has been reported as a taste enhancer (Kuroda et al. 1997; Boran & Regenstein, 2010; Qi et al., 2020). It is formed when the triple helix structure of collagen is denatured by various factors, one of which is high temperature such as boiling and/or heating (Alfaro et al., 2015; Belitz et al., 2009; Tornberg, 2005) and could be further degraded by other metalloproteinases into smaller peptides or to free amino acids (Belitz et al., 2009). Although consumption of *A. marmorata* is not so popular, it could be further developed and utilized into food for value addition. In the Bicol region, freshwater eels are cooked as *paksiw*, with coconut milk and grilled (personal communication). This means that *paksiw* soup from *A. marmorata* and *A. bicolor pacifica*, which may have high gelatin content, can have an enhanced taste.

The fish muscle, skin, scale, bones, and fins were reported as sources of collagen (Jafari et al., 2020; Sikorski & Borderias, 1994; Yoshinaka et al., 1988) which is abundantly contained in the skin, scale, bones, and fin (Yoshinaka et al., 1988). The head and tail part of *A. Anguilla* reported having seemingly higher collagen content than the trunk (Cao et al., 2020). The total collagen in the ordinary muscle in the dorsal part of the trunk of fishes belonging to 24 species was about 0.3-2.2% wet tissue and 1.6-12.4% crude protein with *A. japonica* (01.41-1.99% wet tissue; 8.8-12.4% of crude protein) and *Conger myriaster* (2.19% wet tissue; 11% of crude protein) having the highest values (Sato, Yoshinaka, Sato, & Shimizu, 1986). The 76.2% collagen content of *A. japonica* has been accounted for skin, scales, bones, and fins, whereas the whole body only has 6.97% (Yoshinaka et al., 1988). Since *A. marmorata* and *A. bicolor pacifica* are of the same genus as *A. japonica*, it could be speculated that these species may also have high collagen content. *Anguilla anguilla* is traditionally cooked as jellied eels in the United

Kingdom, where chopped eels are just boiled in stock and allowed to set (Righton & Roberts, 2014). *Anguilla marmorata* and *A. bicolor pacifica* cooked as *paksiw* may have a similar case with jellied *A. Anguilla* with soup having high gelatin content. *Anguilla japonica* collagen content was intensively studied; however, there were no studies for *A. marmorata* and *A. bicolor pacifica*. The slightly better evaluation of *A. marmorata* in terms of chewiness than *A. bicolor pacifica* (Ahn et al., 2015) suggests that it has high collagen content. With gelatin having an effect on the thickness of the taste of soup, *A. marmorata* is well-suited for *paksiw*.

Collagen is the most abundant fibrous protein, which is the main component of the extracellular matrix (Alberts et al., 2002, 2015). It has a triple helix structure mainly characterized by the tripeptide Gly-X-Y with X primarily as proline and Y as hydroxyproline which is involved in the trimeric collagen triple helices (Alberts et al., 2002, 2015; Gelse et al., 2003). Hydroxyproline is an amino acid distinct to collagens, hence, commonly used to estimate collagen content in the muscles, skin or bones (Alberts et al., 2002, 2015; Gelse et al., 2003). Among the different types of collagen in the tissues, Type I collagen is the most predominant characterized by having α-chains [$(\alpha1)_2\alpha2$ and $(\alpha1)3$] (Alberts et al., 2002, 2015; Gelse et al., 2003). The subunits of gelatin and collagen, α1 and α2 single chains or crosslinked as beta (β)-chain (crosslinks between two α-chains), and gamma (γ)-chain (crosslinks between three α-chains), are commonly visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) which were reported to have sizes larger than 94 kDa (Cao et al., 2020; Sato et al., 1986; Sato et al., 1994; Sila et al., 2017). SDS-PAGE is a common method for separating proteins by electrophoresis with polyacrylamide gel as a medium and SDS for protein denaturation, which has been used to visualize the collagenous protein fragments in the muscle (Sato et al., 1986) and skin (Sato et al., 1994; Sila et al., 2017) of freshwater eels. This method

was also used to characterize protein fragments present in cooked fish, and chicken exudates (Qixing et al., 2014; Wattanachant et al., 2005), soups (Hu et al., 2020; Zhang et al., 2013) and broth (Qi et al. 2020), wherein gelatin and other water-soluble proteins were observed.

Precise fish species identification is an important aspect in food processing and development. Polymerase chain-reaction-restricted fragment length polymorphism (PCR-RFLP), one of the fast and reliable techniques, was used to identify 15 known *Anguilla* species (Aoyama et al. 2000; Lin et al. 2002; Watanabe et al. 2005; Chapter 3).

Therefore in this study, species of freshwater eels used for *paksiw* were confirmed by PCR-RFLP. Furthermore, we determined the presence of gelatin in the cooked *paksiw* soup (PS) made from *A. marmorata* and *A. bicolor pacifica* by SDS-PAGE. In addition, gelatin contribution of the skin, muscles, and bones in the soup was investigated. Further, the gelatinous and non-gelatinous proteins were also visualized in the uncooked *paksiw* soup of *A. marmorata* and *A. bicolor pacifica*.

5.2 Materials and Methods

5.2.1 Reagents

Sodium dodecyl sulfate (SDS; Wako, Japan), acrylamide, ammonium persulfate (APS), N,N,N',N' tetramethylethylenediamine (TEMED; Nacalai Tesque, Japan), Sample, Buffer Solution without 2-ME(2x) (Nacalai Tesque, Japan), Bovine Serum Albumin (BSA), Gelatin, Coomassie Brilliant Blue R-250 were used for analysis.

5.2.2 Freshwater eel samples

Two individuals of *A. marmorata* (42.8 cm, 68 cm; 167.5 g, 700.8 g) were caught upstream of Teima River, Nago City, Okinawa, on March 17, 2021 (Fig. 5.1a). Cultured

A. bicolor pacifica (48-53 cm; 200.9-241.2 g) were procured from Bric's ECO JAPAN, Hyogo, Japan (Fig. 5.1b). Live samples were sent to the laboratory and were processed upon arrival. Once in the laboratory, the eels were placed in ice before length and weight measurements, removing gut, head, and tail. The edible portion, 69.4-79.9%, were washed with tap water, placed in a resealable bag, and stored in the freezer for *paksiw* preparation. Dorsal fins were cut and preserved in 70% EtOH for subsequent analysis.

5.2.3 DNA extraction, PCR amplification, and RFLP analysis

DNA was extracted from approximately 25 mg dorsal fin using FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., MI, USA). The extracted DNA was quantified using a nano spectrophotometer (NanoVue, GE). The COI target fragment gene was amplified using the designed primers 5503F1 (5'-CCGCTTAAACATTCAGCC-3') and 7138R1 (5'-GGGGGTTCAATTCCTTCC-3'). PCR reaction was performed in a 25 µl mixture containing 1.0 µl DNA template, 2.5 µl 10× buffer [magnesium (Mg2+) plus], 2.0 µl 2.5 mM deoxynucleoside triphosphate, 1.25 µl 10 µM each primer, 0.125 µl Taq polymerase (Takara Bio Inc., Shiga, Japan), and double-distilled water. Thermal cycler profile included initial denaturation of 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50°C for 30 s, elongation at 72 °C for 60 s, and final extension of 72 °C for 10 min. The PCR amplicons were visualized on 1% agarose Tris-acetate-EDTA (TAE) gel electrophoresis with GelRed Nucleic Acid Gel Stain (Biotium, CA, USA). The PCR products were used for restriction digestion by *Msp* I or *Dde* I protocol (Takara Bio, Japan) protocol. The RFLP patterns were visualized in a 2% agarose Tris-Borate-EDTA (TBE) gel electrophoresis then photographed. RFLP patterns were used for species identification.

5.2.4 Preparation of paksiw

Paksiw for SDS-PAGE analysis was prepared by downscaling the eel samples and water according to the procedure of Tiangson-Bayaga & Deveza (2005) with some modifications. A slice of the middle and tail regions of the eel was weighed. The skin, muscles, and bones were separated and then weighed for computation of the percentage composition of the eel parts used as the basis for the weight of the samples computed for a target of 1,000 mg (refer to Table 5.1 for the computations). The total weight for the cooked or uncooked samples was multiplied by 1.4 for the volume of the water to be added separately in each of the freshwater eel parts.

The uncooked PS was prepared by homogenizing the eel parts using a micropestle. It was then centrifuged at a speed of 7,000 x g for 30 mins at 5 °C. For the cooked samples, eel parts in water were heated for 13 minutes in a heat block at 100 °C, cooled in a water bath, then centrifuged at a speed of 7,000 x g for 30 mins at 20 °C. Supernatants were pipetted out and transferred in a separate microcentrifuge tube and hereinto referred to as the uncooked and cooked PS. A 100-uL each of the supernatants of skin, muscles, and bones were mixed for the uncooked and cooked samples to represent the mixed samples. Soups were stored in the freezer for subsequent analysis. Cooked PS samples were re-heated in a heat block at 37 °C for 60 s to liquefy the gelled soup prior to any analysis.

5.2.5 Protein concentration determination

One (1) uL of each soup sample was added to 200 uL of Coomassie reagent for indirect protein content determination by the standard microplate protocol of Coomassie Protein Reagent Assay Kit (Pierce). A blank and 1-5 uL of 1 mg/mL BSA or gelatin in 200 uL of Coomassie reagent were used as standard. The assay with gelatin standard and adjusted gelatin-dye ratio were as follows 1:20, 1:10, 1:67, 1:5, 1:4.

The measured absorbance of the BSA or gelatin standard volume added to 200 uL Coomassie reagent was corrected by subtracting all absorbances with the blank. The standard curve equation was generated by regression analysis using Microsoft Office Excel Data Package. The protein concentration (expressed in mg/mL) of the sample was computed in Microsoft Excel using the formula below:

$$x = \frac{\mathrm{y} - \mathrm{b}}{\mathrm{m}}$$

where : x= amount of proteins in 1uL

b= 0

y= corrected absorbance

m= slope from the standard curve equation

5.2.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was carried out using the method of Laemmli (1970). Three (3) uL of mixed samples and one (1) uL each for the skin, muscles and bones were mixed with a ratio of 1:1 (v/v) with the SDS-PAGE sample buffer (Nacalai Tesque, Japan). Samples were then loaded into the gel made of 4.5% stacking and 7.5% or 10% separating gels. Then subjected to electrophoresis using a mini slabsize vertical apparatus (ATTO, Japan) run at a constant voltage of 300 volts and current of 25 mA for 40-45 minutes. Electrophoresis run was stopped when the dye buffer front is approximately 1-2 cm from the bottom of the gel. Gels were then stained 0.4% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid for 1.5 hr, decolorized with 10% acetic acid for 1 hr (or until the background is clear). Proteins bands were visualized in a light viewer (Hakuba, Japan) and then photographed.

The molecular weight-stained protein bands were determined by plotting of log (molecular weight) of protein as a function of their migration distance (relative mobility)

in the SDS-PAGE (Dewi, 2002). A molecular weight standard (TEFCO) containing myosin (205 kDa), ß-galactosidase (116 kDa), Phosphorylase b (97.4 kDa), BSA (69 kDa), glutamic dehydrogenase (55 kDa), lactic dehydrogenase (36.5 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa), and insulin B chain (3.5 kDa) was used.

5.3 Results

5.3.1 Species identification

The gel electrophoretic pattern for the seven individuals of freshwater eels is shown in Fig. 5.2. The *Msp* I digestion showed lanes 6-7 have a singlet between 800 bp and 1,00 bp and a doublet between 300 bp and 400 bp, which is similar to the expected pattern of *A. marmorata* Lanes 1-5 have a doublet between 700 bp and 1,000 bp which is similar to the expected pattern for *A. bicolor pacifica* or *A. bicolor bicolor*. The *Dde* I digestion pattern in lanes 6-7 has three bands between 600-700 bp, 400-500 bp, and 200-300 bp. Lanes 1-5 have less than 1,000 bp and doublet between 200-300 bp.

5.3.2 Protein composition by SDS PAGE analysis

The cooked PS of *A. marmorata* (Fig. 5.3a, 5.4a) and *A. bicolor pacifica* (Fig. 5.3b, 5.4b) were observed to have bands larger than 97.5 kDa (lanes 1-3). On the contrary, wherein bands less than 97.4 kDa were mostly found in the uncooked (lanes 5-8) *A. marmorata* (Fig. 5.3a, 5.4a) and *A. bicolor pacifica* (Fig 5.3b, 5.4b).

Anguilla marmorata (Fig. 5.3a) cooked skin PS (lane 2) has seven bands (band #1-7; 112-289 kDa; Table 5.2) which were also observed in mixed PS (lane 1). The muscle PS (lane 3) has four bands (band #3-4, 6,8; 90 kDa, 116 kDa; 215-233 kDa; Table 5.2). The skin PS (lane 2) has high-intensity bands larger than 97.4 kDa than the muscles

PS (lane 3) of *A. marmorata* (Fig. 5.3a). A band around 90 kDa was observed in the cooked muscle PS (lane 3). No visible band were observed in the cooked PS from bones (lane 4). The low molecular weight bands around 42, 44, 28, 27 kDa (band #10-11; 17-18; Table 5.3) were observed only in the cooked muscle (lane 3) PS of *A. marmorata* (Fig. 5.4a)

Eight bands were observed in the cooked *A. bicolor pacifica* PS (Fig. 5.3b) with seven bands larger than 97.4 kDa (band #1-4; 110-297 kDa; Table 5.2) and one band around 89 kDa (band #4). The skin PS (lane 1) has seven bands (band #1-7; 110-297 kDa), four bands (band #3-4, 6, 8; 89 kDa, 119 kDa, 212-229 kDa) in muscle PS (lane 3) and no bands in bone PS (lane 4). *A. bicolor pacifica* (Fig. 1a) cooked skin PS (lane 2) have the high-intensity bands larger than 97.5 kDa than the PS from muscle (lane 3). The low molecular weight bands with sizes 41, 40, 21, and 20 kDa (band #12-13, 20-21; Table 5.3) were observed only in the muscle (lane 3) PS of *A. bicolor pacifica* (Fig. 5.4b).

Eight bands less than 55 kDa were observed in the uncooked PS of *A. marmorata* (lanes 5-8) (Fig. 5.4a). The muscle PS (lane 7) have eight bands (band #9-10; 12-17; 47-44 kDa, 25-38 kDa; Table 5.3). Whereas the skin PS (lane 6) has three bands (band #9-10, 12; 44-47 kDa, 38 kDa) with very low intensity. No bands were observed in bones PS (lane 8).

Anguilla bicolor pacifica uncooked PS (lanes 5-8) has bands less than 97.5 kDa (Fig 5.4b). Eleven bands (band #7-11, 14-19; 43-86 kDa, 24-37 kDa; Table 5.3) less than 97.5 kDa were mostly observed in the uncooked muscle PS of *A. bicolor pacifica* (lane 5; Fig 5.4b). The bands with sizes 30-37 kDa (band #14-16) have the highest intensity. In contrast, very faint bands around 52 kDa and 44 kDa (band #9-10) were observed for the skin (lane 6) and bones PS (lane 8).

5.3.3 Protein concentration

Among the *A. marmorata* parts, the cooked muscle soup of *A. marmorata* (14.78 ± 0.45 mg/mL) have the highest concentration of proteins, followed by the skin (5.37 ± 0.83 mg/mL) and bones (4.31 ± 0.95 mg/mL) (Table 5.4). Similarly, the cooked muscle soup of *A. bicolor pacifica* (21.18 ± 1.36 mg/mL) has the highest concentration of proteins, followed by the bones (4.20 ± 0.42 mg/mL) and the skin (3.88 ± 0.07 mg/mL) (Table 5.4).

The uncooked muscle soup of *A. marmorata* (62.27 ± 4.81 mg/mL) have the highest concentration of proteins, followed by the skin (15.88 ± 0.94 mg/mL) and bones (11.06 ± 1.47 mg/mL) uncooked soups (Table 5.4). Whereas the uncooked *A. bicolor pacifica* muscles (51.77 ± 0.13 mg/mL) have the highest concentration of proteins, followed by the bones (14.20 ± 1.95 mg/mL) and skin (8.59 ± 0.38 mg/mL) (Table 5.4).

Using gelatin as the standard, the protein concentration of the *A. marmorata* cooked muscles PS were highest (34.46 ± 2.97 mg/mL), followed by the skin (23.85 ± 0.27 mg/ mL) and then the bones (23.59 ± 0.22 mg/mL). Similarly, *A. bicolor pacifica* cooked muscle PS (34.03 ± 0.60 mg/mL) has the highest concentration of proteins, followed by the bones (21.48 ± 0.38 mg/mL), and the skin (20.26 ± 0.62 mg/mL).

5.4 Discussion

5.4.1 Species identification

The *Msp* I and *Dde* I digestion patterns in lanes 6-7 indicated that these two individuals were *A. marmorata*. The individuals that showed the RFLP patterns in lanes 1-5 could either be *A. bicolor pacifica* or *A. bicolor bicolor* (*Msp I*, Fig. 5.2; Fig. 3.1a; Chapter 3) as indicated by the doublet between 700 bp and 1,000 bp. However, the *Dde* I digestion confirmed that these individuals were *A. bicolor pacifica*.

5.4.2 Protein composition by SDS-PAGE analysis

The collagen was reported to have α, β and γ subunits which may have sizes larger than 94 kDa as observed in the SDS-PAGE patterns (Sato et al. 1986, 1994; Sila et al. 2017; Cao et al. 2020). The cooked PS of *A. marmorata* and *A. bicolor pacifica* were observed to have protein bands larger than 97.4 kDa, which could be the α, β and γ chains and none for the uncooked PS of *A. marmorata* and *A. bicolor pacifica*. The cooked skin PS has the same number of bands larger than 97.4 kDa, with the mixed one indicated that almost collagen denatured fragments were from the skin. The observed bands around 289 kDa (band #1), 275 kDa (band #2), 209 kDa (band #5), and 112 kDa (band #7) in the cooked skin soup (lane 2) suggested that these were only from the skin. Whereas the bands around 116 kDa (band #6), 215 kDa (band #4), and 233 kDa (band #3) were both from the skin and muscles. The muscles were the origin of the band around 90 kDa (band #8; Fig. 5.2a) and the low molecular weight bands around 42, 44, 28, 27 kDa (band # 10-11; 17-18 (lane 3) PS (Fig. 5.3a).

In *A. bicolor pacifica* cooked PS, the observed bands larger than 97.4 kDa mainly were from the skin. Bands observed around 110 kDa (band #7), 209 kDa (band #5), 263, and 297 kDa (bands #1-2) were only derived from the skin (lane 2; Fig. 5.2a). The bands around 119 kDa (band #6), 229 kDa (band 3 and 212 kDa (band #4) were both from the skin (lane 2) and muscle (lane 3; Fig. 5.2a). The band around 89 kDa (bands #8) originated from the muscles (lane 3).

Two bands around 275 kDa and 289 kDa observed in the cooked PS of *A. marmorata* (Fig. 5.2a) were of higher intensity than in *A. bicolor pacifica* PS, which is of very low intensity (263 kDa and 297 kDa; bands #1-2; Fig. 5.2b). In addition, the bands found within the vicinity of 205 kDa and 116 kDa in the cooked *A. marmorata* PS (Fig. 5.2a) seem to have higher staining intensity than the ones observed in the cooked PS of

A. bicolor pacifica (Fig. 5.2b). Although no bands were found in bones (lane 4) in the 7.5% gel for *A. marmorata* and *A. bicolor pacifica* cooked PS (Fig. 5.3b), a very low intensity band around 116 kDa was observed in 10% gel (Fig. 5.4b) for *A. bicolor pacifica* PS and almost neglible in *A. marmorata*. A high percentage gel can visualize bands with very low intensity that cannot be resolved by gels having low percentage; hence the appearance of bands in the bones for the cooked *A. marmorata* and *A. bicolor pacifica*. The bands observed between 36.5 kDa and 55 kDa in *A. marmorata* were less intense than those observed in the *A. bicolor pacifica* PS. Two bands less than 29 kDa observed in the *A. bicolor pacifica* PS were also observed in *A. marmorata*, but the intensity was too low. These low molecular weight bands were derived from the muscles (lane 3; Fig. 5.3a,b) and were the most intense band than the collagen-related bands larger than 97.4 kDa. These were only observed in the muscles and none in the skin and bones. Muscles are mostly made up of myofibrillar proteins (Tornberg, 2005; Belitz et al., 2009) that may have migrated from the freshwater eel meat to the soup and could be the troponin T, troponin I, and beta-actin as similarly observed in the chicken muscle broth (Qi et al. 2017). In addition, no bands less than 55 kDa were observed due to the absence of myofibrillar proteins in the bone structure. However, it was revealed that the skin is the origin of most of the gelatin in the cooked Pakiw soup of *A. marmorata* and *A. bicolor pacifica*

The presence of bands within the vicinities of 116 kDa and 205 kDa in the cooked PS of *A. marmorata* and *A. bicolor pacifica* suggested that these were the denatured collagen (α, β, and γ) by boiling which maybe gelatin. Compared to collagen, gelatin is water-soluble (Tornberg, 2005; Belitz et al., 2009), hence present in the cooked soup. However, low molecular weight gelatin-related peptides were not observed since enzymes that could further degrade gelatin may have been inactivated due to boiling for

more than ten minutes. It has been reported that enzymes could be inactivated very fast (~1 min) by heating at 100 °C (Belitz et al., 2009; Ahnoff et al. 2015) for more than ten minutes (Ahnoff et al. 2015). Although Anguillids were reported to have high collagen content (Sato et al. 1986), collagen was only denatured and solubilized to gelatin during the heating/cooking process. Gelatin, denatured by heating or cooking, is transferred (or migrated) in the soup. Both skin and muscles were sources of gelatin in the cooked PS; however, most of the gelatin content was derived from the skin. Therefore, gelatin in the cooked PS of *A. marmorata* and *A. bicolor pacifica* can enhance its taste.

Most of the bands less than 55 kDa observed in the uncooked *A. marmorata* PS were contributed by the muscles. Similarly, the muscles were also the source of most bands less than 97.4 kDa in the *A. bicolor pacifica* uncooked PS.

Bands larger than 55 kDa observed in *A. bicolor pacifica* PS (69 kDa, 86 kDa; band #6-7; Fig. 5.3b) were not observed in *A. marmorata* PS (Fig. 5.3a). Most bands observed in the uncooked *A. marmorata* PS were less than 55 kDa (lane 5; Fig. 5.3a). The bands observed in uncooked *A. marmorata* PS (lane 5; Fig. 5.3a) within the vicinity of 36.5 kDa were of higher intensity than those found in *A. bicolor pacifica* (lane 5; Fig. 5.3b). The water-soluble proteins or sarcoplasmic proteins which may be the glycolytic pathway enzymes creatine kinase and myoglobin (Tornberg 2005), which were derived from the muscles, could be the bands observed in the uncooked soup of *A. marmorata* and *A. bicolor pacifica*.

5.4.3 Protein concentration

Among the cooked PS from eel parts, the cooked muscle PS of *A. marmorata* (14.78 ± 0.45 mg/mL) has the highest protein concentration (Table 5.4). The skin PS has a lower protein concentration than the muscle soup (Table 5.4). A similar case was

observed for the cooked PS of *A. bicolor pacifica*, wherein the muscle (21.18 ± 1.36 mg/mL) has the highest concentration of proteins among the eel parts (Table 5.4). The skin PS, also has a lower protein concentration than the muscle soup (Table 5.4). Both muscle PS of *A. marmorata* and *A. bicolor pacifica* has the highest protein concentration among the eel parts.

The uncooked muscle PS of *A. marmorata* (62.27 ± 4.81 mg/mL) has the highest protein concentration (Table 5.4). Similarly, the uncooked muscle PS of *A. bicolor pacifica* (51.77 ± 0.13 mg/mL) also has the highest protein concentration among all the eel parts (Table 5.4). Thus, both muscle PS of *A. marmorata* and *A. bicolor pacifica* has the highest protein concentration among the eel parts. This may be due to the presence of not only the gelatinous but also the non-gelatinous proteins as observed in the SDS-PAGE patterns (Fig. 5.4 a, b).

The cooked PS of *A. marmorata* (9.06 ± 0.59 mg/mL) and *A. bicolor pacifica* (10.82 ± 1.09 mg/ml) have lower protein concentrations than the uncooked ones, 45.80 ± 8.8 mg/mL, and 34.86 ± 0.66 mg/mL, respectively. Low sensitivity of gelatinous proteins using Coomassie protein assay with BSA as the standard was reported (Lopez et al. 1993) and could be attributed to the lower concentration of cooked skin soup than the muscle, which may be due to the high presence of gelatinous proteins in the skin soup which was underestimated.

Using gelatin as the standard, high values of protein concentration was observed. Still, the cooked muscle PS of *A. marmorata* (34.46 ± 2.97 mg/mL) has the highest concentration amongst the eel parts. On the other hand, the skin PS (23.85 ± 0.27 mg/mL) has a lower protein concentration than the muscle PS. A similar case was observed in *A. bicolor pacifica* cooked muscle PS (34.03 ± 0.60 mg/mL). It has a higher protein concentration than the skin (20.26 ± 0.62 mg/mL).

The cooked muscle PS of *A. marmorata* and *A. bicolor pacifica* have the highest protein concentration among the eel parts. On the other hand, the cooked skin PS of *A. marmorata* and *A. bicolor pacifica* have lower protein concentration than the muscles. The higher cases were observed in both protein assays with BSA or gelatin as the standard. However, protein concentration in the cooked samples with gelatin as the standard was higher than that of the values using BSA standard. Nevertheless, the cooked muscle PS which still has the highest protein concentration may mean that the non-gelatinous proteins are highly sensitive to the Coomassie protein assay.

Bands observed in the cooked PS of *A. marmorata* and *A. bicolor pacifica* were mostly the gelatin fragments α, β, and γ chains, suggesting that collagens from the tissues of the freshwater eels were solubilized when cooked and liberated to the soup. The α chains observed within the vicinity of 116 kDa may be the α1 and α2 with α1 as the upper band having higher staining intensity than α2 in the lower band. The higher number of α1 molecules than the α2 makes its mobility in the gel slower thereby; bands were observed as the upper band within 116 kDa and α2 as the lower band. Although theoretically, the lengths of the α1 and α2 subunits were almost the same, their difference in the amino acid composition (Ayad et al., 1998) may influence the mobility and its post-translational glycosylation on the characteristic of the α chain. The higher band intensity of α1 than α2 could be due to higher number of α1 molecules due to the presence of the molecular species $(\alpha1)_2\alpha2$ and $(\alpha1)_3$ in the tissues (Haralson & Hassell 1995; Alberts et al., 2002, 2015). In the case of the two β bands observed within the vicinity of 205 kDa, the lower band has a higher intensity than the upper band. This may be due to the selectivity in the crosslinking of the α chain. If the α1 chains are equally crosslinked, there may be a high possibility of α1 dimers due to many polypeptides. However, the intensity of the lower β band, which is higher than that of the upper β band, indicated otherwise.

Although protein concentration of the cooked skin PS is lower than that of the muscle using the Coomassie Assay (Table 5.4), staining intensity of the bands of skin (lane 2, Fig. 5.3-4 a,b) is higher than the muscles (lane 3, Fig. 5.3-4 a,b) which may suggest the opposite case. The quantification of protein concentration by densitometric analysis of the bands in the 10% gel of *A. marmorata* PS showed that the cooked skin PS has the highest concentration (4.17 mg/mL) among the eel parts, muscles (1.65 mg/mL) being the second, and lastly bones (0.27 mg/mL). A similar case was observed for cooked *A. bicolor pacifica* PS with skin (4.80 mg/mL) having higher protein concentration followed by muscles (2.98 mg/mL) then the bones (0.37 mg/mL). The estimation of protein concentration based the densitometric analysis is opposite with the values determined using the Coomassie protein assay. Between CBB G-250 and CBB R-250, the CBB R-250, which used for staining SDS-PAGE gels, is reported to be more sensitive than CBB G-250 (Bio-Imaging Systems, n.d.). Whereas, sensitivity of gelatin to CBB G-250 is very low. Hence, the sensitivity of gelatin to the different dyes used for protein assay (CBB G-250) and densitometry (CBB R-250) is the reason why opposite cases for the skin and muscle concentration differences.

Presence of the gelatin can only be observed in the cooked PS of *A. marmorata* and *A. bicolor pacifica*. Most of the gelatin were derived from the skin as suggested by the intensity of the bands. However, protein concentration determined by the Coomassie protein assay with BSA as the standard showed higher concentration of the muscles than the skin due to high sensitivity of the non-gelatinous proteins. Hence the use of gelatin as the standard for protein concentration yet showed similar trend with muscles having higher protein concentration than the skin. However, confirmation by densitometric analysis revealed the higher protein concentration than muscles. The difference in the CBB dyes used for the protein assay (CBB G-250) and staining for SDS-PAGE (CBB R-

250) may be attributed to such discrepancy since CBB R-250 is more sensitive than the former. The slight differences of the protein concentrations of the cooked PS of *A. marmorata* and *A. bicolor pacifica* as reported by Coomassie protein assay (BSA and gelatin standards) and the densitometric analysis suggest that gelatin content of the soup of *A. marmorata paksiw* is comparable with *A. bicolor pacifica*. High protein content (including gelatin) may result in a richer, thicker taste of the soup, hence will be further investigated.

Table 5.1 Computed weights of samples used for downscaling of the cooked and uncooked soup of *paksiw* made from *A. marmorata* and *A. bicolor pacifica* which was added with 1.4 volumes of water.

Freshwater eel parts	Species					
	A. marmorata			***A. bicolor pacifica***		
	Percentage composition (%)	**Cooked**	**Uncooked**	**Percentage composition (%)**	**Cooked**	**Uncooked**
Skin	14.5	145.2 mg	145.2 mg	15.4	154.5 mg	154.1 mg
Muscle	76.3	763.2 mg	763.3 mg	73.1	730.6 mg	730.2 mg
Bones	9.1	92.2 mg	90.6 mg	11.5	112.9 mg	115.5 mg
Total	99.9	1,000.6 mg	999.1 mg	100	998.0 mg	999.8 mg

Note: Computation were based on the percentage composition of each of the parts in a 1,000 mg target weight of freshwater eel

Table 5.2 Estimated band sizes of fragments observed in 7.5% gel for the cooked *paksiw* soup of *A. marmorata* and *A. bicolor pacifica*

Species	Band	Estimated molecular weight	
A. marmorata	1	289	γ
	2	275	γ
	3	233	β
	4	215	β
	5	209	β
	6	116	α
	7	112	α
	8	90	
A. bicolor pacifica	1	297	γ
	2	263	γ
	3	229	β
	4	212	β
	5	209	β
	6	119	α
	7	110	α
	8	89	

Table 5.3 Estimated band sizes of fragments observed in 10% gel for the cooked and uncooked *paksiw* soup of *A. marmorata* and *A. bicolor pacifica*.

Species	Band	Estimated molecular weight (kDa)	
		Cooked PS	Uncooked PS
A. marmorata	1	291	
	2	258	
	3	207	
	4	184	
	5	113	
	6	107	
	7	98	
	8	73	
	9		47
	10	44	44
	11	42	
	12		38
	13		36
	14		31
	15		29
	16		27
	17	28	25
	18	27	
A. bicolor pacifica	1	282	
	2	229	
	3	205	
	4	116	
	5	95	
	6	88	
	7		86
	8		69
	9		52
	10		44
	11		43
	12	41	
	13	40	
	14		37
	15		34
	16		30
	17		29
	18		27
	19		24
	20	21	
	21	20	

Table 5.4 Protein concentration of the cooked and uncooked *paksiw* soup made from *A. marmorata* and *A. bicolor pacifica*.

Standard used for Coomassie Protein Assay	*Paksiw* Soup Samples	*A. marmorata*		*A. bicolor pacifica*	
		Cooked	**Uncooked**	**Cooked**	**Uncooked**
BSA	Mixed	9.06 ± 0.59	45.80 ± 8.80	10.82 ± 1.09	34.86 ± 0.66
	Skin	5.37 ± 0.83	15.88 ± 0.94	3.88 ± 0.07	8.59 ± 0.38
	Muscles	14.78 ± 0.45	62.27 ± 4.81	21.18 ±1.36	51.77 ±0.13
	Bones	4.31 ± 0.95	11.06 ± 1.47	4.20 ± 0.42	14.20 ± 1.95
Gelatin	Mixed	27.92 ± 1.31		30.41 ± 0.32	
	Skin	23.85 ± 0.27		20.26 ± 0.62	
	Muscles	34.46 ± 2.97		34.03 ± 0.60	
	Bones	23.59 ± 0.22		21.48 ± 0.38	

Note: Protein concentrations are expressed in mg/mL.

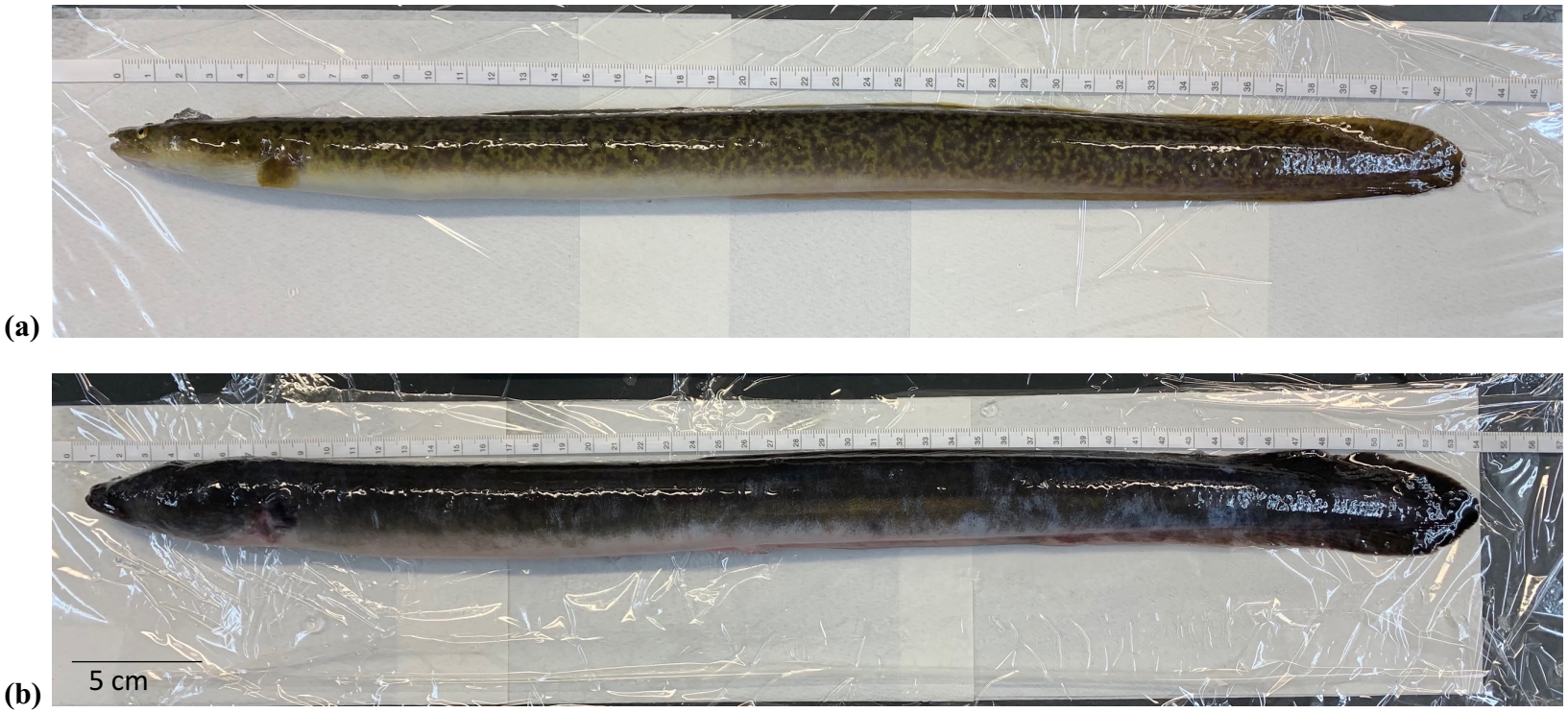


Fig. 5.1 Live (**a**) *Anguilla marmorata* and (**b**) *Anguilla bicolor pacifica*; scale bar- 5 cm.

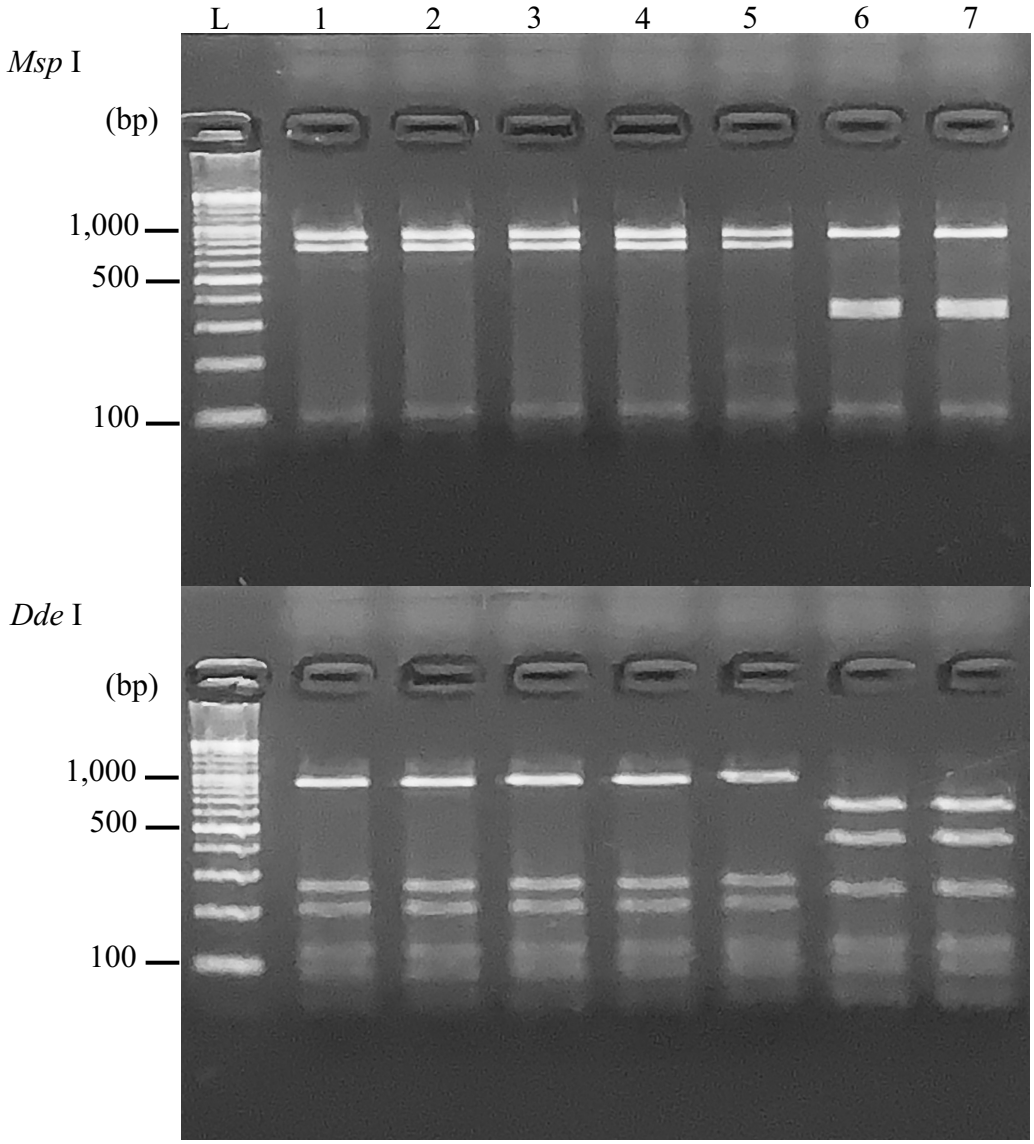


Fig. 5.2 Gel electrophoretic patterns of the digested PCR amplicons by restriction enzymes, *Msp* I and *Dde* I.
L- ladder; Lanes 1-5 for *A. bicolor pacifica*; Lanes 6-7 for *Anguilla marmorata* (lanes 6-7); bp- base pairs

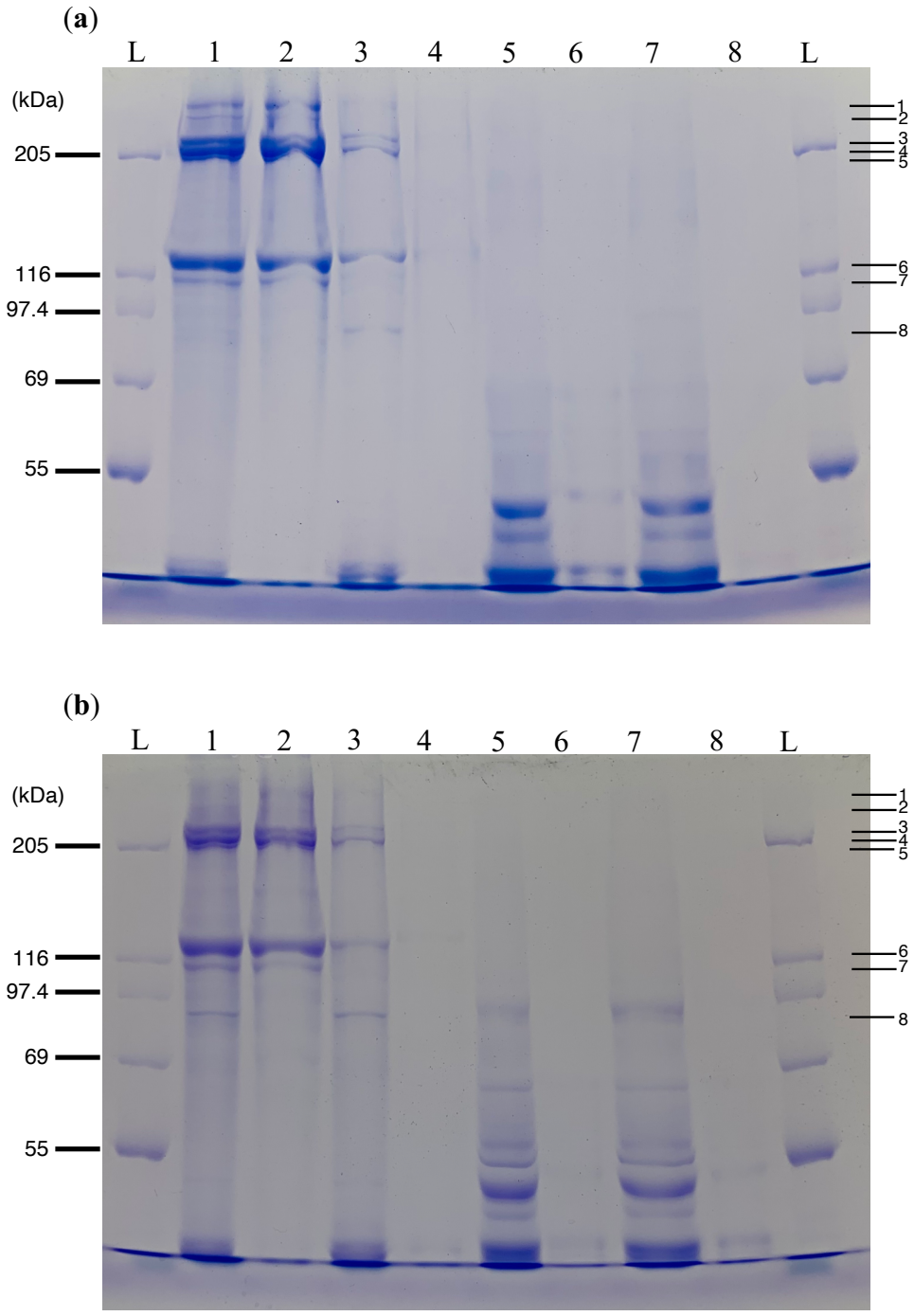


Fig. 5.3 SDS-PAGE pattern of cooked (lanes 1-4) and uncooked (lanes 5-8) *paksiw* soup made from (**a**) *A. marmorata* and (**b**) *A. bicolor pacifica* in 7.5% acrylamide gel. L- ladder; Lanes: 1, 5- mixed, 2, 6- skin, 3, 7- muscle, 4, 8- bones; kDa- kilodalton

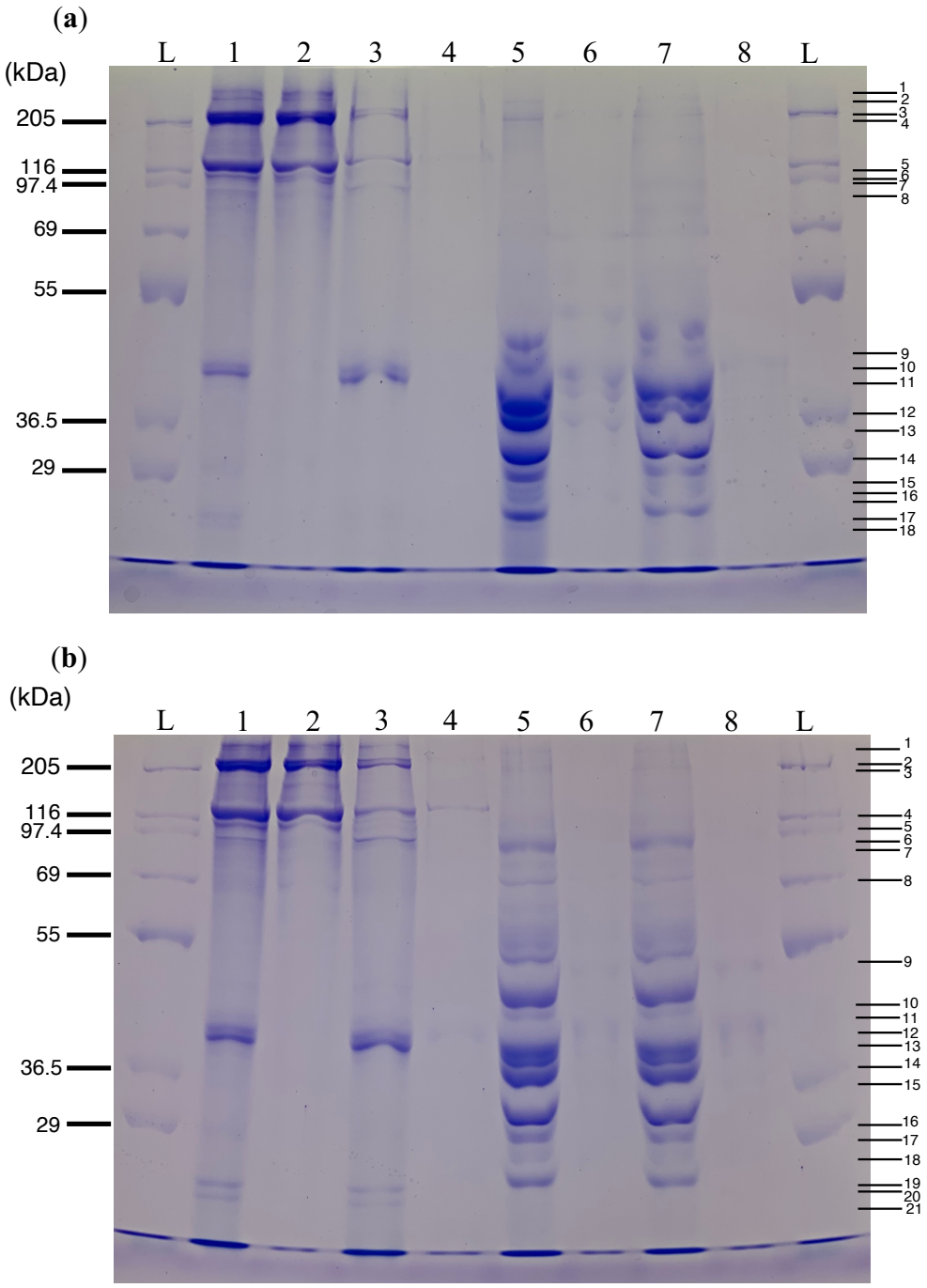


Fig. 5.4 SDS-PAGE pattern of cooked (lanes 1-4) and uncooked (lanes 5-8) *paksiw* soup made from (**a**) *A. marmorata* and (**b**) *A. bicolor pacifica* in 10% acrylamide gel. L- ladder; lanes: 1, 5- mixed, 2, 6- skin, 3, 7- muscle, 4, 8- bones; kDa- kilodalton

Chapter 6 General Discussion

Freshwater eels are a commercially important commodity in the Philippines. *Anguilla marmorata*, *Anguilla bicolor pacifica*, *Anguilla japonica*, *Anguilla bicolor bicolor* and *Anguilla luzonensis* are being exported to the traditionally eel consuming countries in the East Asia. Most of the Anguillids recruited in the Philippines were reported to have spawning grounds in the North Equatorial Current (NEC) and transported by the NEC bifurcates, hence the possibility of recruitment in Lagonoy Gulf, Bicol region which is near the NEC bifurcates. *Anguilla marmorata* is the most abundant species collected in the Philippines. However, *A. bicolor pacifica* is preferred in the export market due to reportedly similar taste and texture with *A. japonica*. However, studies have shown the comparable nutritional composition and superior preference test of *A. marmorata* compared to *A. bicolor pacifica*. Collagen content of *A. japonica* which is commonly cooked as *kabayaki* is high, which can be speculated to be also high in *A. marmorata* and *A. bicolor pacifica* given the same genus. Collagen is denatured when fish meat is cooked and converted to gelatin which is reported as a taste enhancer. Thus, *A. marmorata* have the potential for food development and innovation which can be explored. However, no information available for the freshwater eel consumption and collection in the Bicol region, hence documented.

Consumption of freshwater eels in the Philippines is not that popular in this generation. In the Bicol region, freshwater eels are generally caught for home consumption only during special occasions. Mainly freshwater eels are grilled (*inihaw*), cooked with vinegar (*paksiw*), or with coconut milk (*ginataan*). The collection of freshwater eels uses gears depending on their life stage. Adult freshwater eels are caught using hook and line, speargun, pushnet, electrofishing, rock mounds, and traps. On the

other hand, glass eels were commonly collected using a modified fyke net (*kubong*). These freshwater eel collection gear have also been reported to be used in the Philippines. Although not famous for consumption, the abundance of low valued freshwater eel species in the Philippines may be utilized for food development that could create new market and provide alternative livelihood for the fishers. However, no data are available on the species recruited and its proportion in the Bicol region especially Lagonoy Gulf which is geographically located near the spawning grounds of Anguillids, hence was investigated.

Anguillids recruited in the tributaries along Lagonoy Gulf were initially identified by morphology limited to pigmentation patterns. *Anguilla japonica* was easily separated from the group due to absence of pigmentation. In addition, *A. bicolor pacifica* individuals seemed to be almost distinguished by the dense pigmentation on the tail reaching the caudal tip. However, due to the difficulty in the distinction of *A. marmorata* from *A. luzonensis* with both having patterns on the tail not reaching the tip of the caudal fin, the 4801 glass eels collected were mostly grouped as *A. marmorata*, followed by *A. bicolor pacifica* and a negligible quantity of *A. japonica*. However, PCR-RFLP analysis by *Msp* I and/or *Dde* I followed by DNA sequencing only, when necessary, found *A. marmorata*, *A. bicolor pacifica*, *A. luzonensis*, and *A. celebesensis*. Species composition based on molecular analysis revealed the dominance of *A. marmorata*, followed by that of *A. luzonensis*, *A. bicolor pacifica* as the third frequent occurring species in and rare recruitment of *A. celebesensis*. The existence of *A. luzonensis* and *A. celebesensis* in Lagonoy Gulf was only confirmed using molecular analysis. Therefore, pigmentation patterns used for the distinction of the pigmented glass eels were clarified for *A. marmorata*, *A. luzonensis* and confirmed for *A. bicolor pacifica*. The typical pigmentation pattern used for the identification of *A. bicolor pacifica* was found to be also exhibited by

A. celebesensis, hence this pattern cannot be used to distinguish these two species. However, the pigmentation pattern on the tail not reaching the tail tip was observed both for *A. marmorata* and *A. luzonensis*. Based on our observation, patterns c and e exhibited only by *A. marmorata* and *A. luzonensis*, respectively. However, patterns d and g is still unclear since these were mostly exhibited by *A. marmorata* and only one individual of *A. luzonensis*. Both *A. marmorata* and *A. luzonensis* exhibited pattern b. Using pigmentation patterns, Anguillids collected in the tributaries along Lagonoy Gulf can only be separated into the group of *A. bicolor pacifica* and *A. celebesensis* (pattern a); and the group of *A. marmorata* and *A. luzonensis* (patterns b-g). The use of pigmentation patterns alone was not enough for distinction of Anguillids in the Philippines; hence molecular analysis is better to be used for precise species identification for a precise estimate of the species composition.

Anguilla luzonensis have limited information on population structure hence was investigated in this study. The occurrence of *A. luzonensis* in Lagonoy Gulf was first reported in this study. Although genetic variability was found in *A. luzonensis* individuals recruited in Comun and Lagonoy rivers, genetic diversity was very low and not significantly different, which was inferred from the partial COI gene fragment. The monophyly of *A. luzonensis* individuals from the Comun and Lagonoy rivers, including the two DNA sequences downloaded from NCBI collected from Cagayan and once from Bato river imply that these individuals share similar genetic materials. The result suggested the possibility of *A.luzonensis* panmixia, however, genetic differences and population structure of this species from Luzon, Mindanao, Philippines, Taiwan, and Okinawa, Ryuku archipelago may be compared and studied in the future. Although no information on its market value, *A. luzonensis* being the second abundant species in Lagonoy Gulf and in Cagayan indicates its potential for utilization. The occurrence of the

high-value species *A. bicolor pacifica*, this study provides information that will help effective management and utilization of freshwater eels. The dominance of the low-value species, *A. marmorata*, also indicated its potential for product development to improve its marketability in the region.

Therefore, we tried to investigate the presence of gelatin, a taste enhancer in the soup of *A. marmorata* and *A. bicolor pacifica* cooked as *paksiw*, one of the famous cuisines in the Philippines. Although the study revealed the presence of gelatin in the soup of *paksiw* made from *A. marmorata* and *A. bicolor pacifica*, samples used for the study were prepared on a small-scale or downscaled version of the cuisine. Further study on the gelatin and its related components must be carried out for the standard or large-scale cooking of *A. marmorata* and *A. bicolor pacifica paksiw*. The gradual increase of temperature while freshwater eel is being cooked may have aided the action of enzymes (exopeptidases; collagenase) to degrade the denatured collagen into small peptides further and free amino acids; hence free and gelatin-bound hydroxyproline may be quantified. Consequently, the total gelatin content may also be estimated by the free and gelatin-bound hydroxyproline. The taste active components could be determined to describe which compounds have been enhanced by the presence of gelatin. Although texture of *A. marmorata* is inferior to *A. japonica*, these species of the same genus may both have high collagen content compared with other fish species belonging to other groups which is an advantage, hence we selected to investigate gelatin. Thus, taste enhancement by gelatin in the *A. marmorata* and *A. bicolor pacifica paksiw* must be confirmed by preference test and compared with other fish species having low collagen content. Our current study revealed that gelatin content of the *A. marmorata* PS is comparable with *A. bicolor pacifica*. Focusing on the effective processing methods of *A. marmorata*, may entice aquaculture owners to grow glass eels until the adult stage that may lead to new products

and create new markets and avenues to improve livelihood of the fishers in the Bicol Region.

Literature Cited

Ahn, J. C., Chong, W.-S., Na, J. H., Yun, H. B., Shin, K. J., Lee, K. W., & Park, J. T. (2015). An Evaluation of Major Nutrients of Four Farmed Freshwater Eel Species (Anguilla japonica, A. rostrata, *A. bicolor pacifica* and *A. marmorata*). *Korean Journal of Fisheries and Aquatic Sciences*, *48*(1), 44–50. https://doi.org/10.5657/kfas.2015.0044

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002). Molecular Biology of the Cell. 4th ed, United Kingdom: Garland Science, Taylor and Francis Group. ISBN 978-0-8153-4072-9

Alberts B, Johnson A, Lewis J, Morgan D, Raff M, Roberts K, Walter P (2015). Molecular Biology of the Cell. 6th ed, United Kingdom: Garland Science, Taylor and Francis Group. ISBN 978-0-8153-4464-3

Alfaro, A. da T., Balbinot, E., Weber, C. I., Tonial, I. B., & Machado-Lunkes, A. (2015). Fish Gelatin: Characteristics, Functional Properties, Applications and Future Potentials. In *Food Engineering Reviews* (Vol. 7, Issue 1, pp. 33–44). https://doi.org/10.1007/s12393-014-9096-5

Ame, E. C., Ayson, J. P., & Ame, R. B. (2013). Status of Elvers Fisheries in Cagayan Province , Luzon , Philippines. *Kuroshio Science*, *7*(1), 41–48.

Aoyama, J, Watanabe, S., Nishida, M., & Tsukamoto, K. (2000). Discrimination of Catadromous Eels of Genus Anguilla Using Polymerase Chain Reaction–Restriction Fragment Length Polymorphism Analysis of the Mitochondrial 16S Ribosomal RNA Domain. *Transactions of the American Fisheries Society*, *129*(3), 873–878. https://doi.org/10.1577/1548-8659(2000)129<0873:doceog>2.3.co;2

Aoyama, J. (2009). Life History and Evolution of Migration in Catadromous Eels (Genus Anguilla). *Aqua-BioScience Monographs*, *2*(1), 1–42. https://doi.org/10.5047/absm.2009.00201.0001

Aoyama, J., Kobayashi, T., & Tsukamoto, K. (1996). Phylogeny of eels suggested by mitochondrial DNA sequences. *Nippon Suisan Gakkaishi*, *62*(3), 370–375.

Aoyama, J., Nishida, M., & Tsukamoto, K. (2001). Molecular phylogeny and evolution of the freshwater eel, genus Anguilla. *Molecular Phylogenetics and Evolution*, *20*(3), 450–459. https://doi.org/10.1006/mpev.2001.0959

Aoyama, J., & Tsukamoto, K. (1997). Evolution of the freshwater eels. In *Naturwissenschaften* (Vol. 84, Issue 1, pp. 17–21). Springer-Verlag. https://doi.org/10.1007/s001140050340

Aoyama, J., Wouthuyzen, S., Miller, M. J., Inagaki, T., & Tsukamoto, K. (2003). Short-Distance Spawning Migration of Tropical Freshwater Eels. *The Biological Bulletin*, *204*(1), 104–108. https://doi.org/10.2307/1543500

Aoyama, J., Yoshinaga, T., Shinoda, A., Shirotori, F., Yambot, A. V., & Han, Y. S. (2015).

Seasonal changes in species composition of glass eels of the genus anguilla (Teleostei: Anguillidae) recruiting to the cagayan river, Luzon Island, the Philippines. *Pacific Science*, *69*(2), 263–270. https://doi.org/10.2984/69.2.8

Arai, T., Limbong, D., Otake, T., & Tsukamoto, K. (2001). Recruitment mechanisms of tropical eels Anguilla spp. and implications for the evolution of oceanic migration in the genus Anguilla. *Marine Ecology Progress Series*, *216*, 253–264. https://doi.org/10.3354/meps216253

Asian Food Network. (2020). *Paksiw na Isda (Milkfish Stewed in Vinegar)*. Retrieved June 3, 2021, from https://asianfoodnetwork.com/en/recipes/cuisine/filipino/paksiw-na-isda.html

Ayad, S., Boot-Handford, R., Humphries, M., Kadler, K., & Shuttleworth, C. A. (1998). Extracellular Matrices. In *The Extracellular Matrix Factsbook* (2nd ed., pp. 11–20). Academic Press.

Belitz HD, Grosch W, Schieberle P (2009). Food Chemistry. 4th ed, Springer, Berlin, Germany. 670-742

Bio-Imaging Systems. (n.d.). *Commassie Brilliant Blue*. Retrieved June 24, 2021, from https://www.berthold-jp.com/pdf/coomassie_blue.pdf

Boquet, Y. (2017). The use and abuse of sea resources. In *Springer Geography* (pp. 301–339). Springer. https://doi.org/10.1007/978-3-319-51926-5_11

Boran, G., & Regenstein, J. M. (2010). Fish gelatin. *Advances in Food and Nutrition Research*, *60*, 119–143. https://doi.org/10.1016/S1043-4526(10)60005-8

Cao, W., Shi, L., & Weng, W. (2020). Histological Distribution and Characterization of Collagen in European eel (Anguilla anguilla) Muscle. *Journal of Aquatic Food Product Technology*, *29*(1), 121–131. https://doi.org/10.1080/10498850.2019.1695694

Castle, P. H. J., & Williamson, G. R. (1974). On the Validity of the Fresh-Water Eel Species Anguilla ancestralis Ege, from Celebes. *Copeia*, *1974*(2), 569. https://doi.org/10.2307/1442564

Cheng, I. C., Liao, J. X., Ciou, J. Y., Huang, L. T., Chen, Y. W., & Hou, C. Y. (2020). Characterization of protein hydrolysates from eel (*Anguilla marmorata*) and their application in herbal eel extracts. *Catalysts*, *10*(2). https://doi.org/10.3390/catal10020205

Crook, V. (2014). *Slipping away: International Anguilla eel trade and the role of the Philippines.* TRAFFIC and ZSL, UK. ISBN 978-1-85850-376-9

Cuvin-aralar, M. L., & Romana-eguia, M. R. (2019). *Nursery Culture of Tropical Anguillid Eels in the Philippines*. *65*. https://repository.seafdec.org.ph/handle/10862/3444

DA-BFAR. (2018). *BFAR On-Line Information System*. Retrieved February 11, 2021, from https://www.bfar.da.gov.ph/profile?id=2

DA-BFAR. (2019). *Philippine Fisheries Profile 2018*. https://www.bfar.da.gov.ph/publication

Dou, S. (2014). Eels in China: Species, Fisheries, Stock Management and Culture. In Katsumi Tsukamoto & M. Kuroki (Eds.), *Eels and Humans* (2nd ed., pp. 117–128). Springer Japan. https://doi.org/10.1007/978-4-431-54529-3_8

Department of Science and Technology- Food and Nutrition Research Institute (DOST-FNRI) (2018). 2018 Menu Guide Calendar: Focusing on Energy and Nutrient Needs of Older Persons through Pinggang Pinoy. Retrieved February 1, 2021, https://fnri.dost.gov.ph/images/images/standardtools/MenuGuideCalendar/2018-MGC.pdf

Ersoy, B., Aksan, E., & Özeren, A. (2008). The effect of thawing methods on the quality of eels (Anguilla anguilla). *Food Chemistry*, *111*(2), 377–380. https://doi.org/10.1016/j.foodchem.2008.03.081

Excoffier, L., & Lischer, H. E. L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, *10*(3), 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Fernandez, D. G. (1988). Culture Ingested: Notes on the Indigenization of Food. *Philippine Studies*, *36*(2), 219–232. http://www.philippinestudies.net

Food and Agriculture Organization. (2009). *FAO - Anguilla japonica*. http://www.fao.org/fishery/docs/DOCUMENT/aquaculture/CulturedSpecies/file/en/en_japaneseeel.htm

Food and Agriculture Organization (FAO). (2020) Global Production statistics: online query. Retrieved July 4, 2020, from URL: http://www.fao.org/figis/servlet/TabLandArea?tb_ds=Production&tb_mode=TABLE&tb_act=SELECT&tb_grp=COUNTRY&lang=en

Foundation for the Philippine Environment (FPE). (2009). *Upper Marikina River Basin Protected Landscape*. 2009–2011.

Gelse, K., Pöschl, E., & Aigner, T. (2003). Collagens - Structure, function, and biosynthesis. *Advanced Drug Delivery Reviews*, *55*(12), 1531–1546. https://doi.org/10.1016/j.addr.2003.08.002

Geronimo, M. C., Cabansag, M. G. S., & Reyes, A. S. (2016). Indigenous Utilization of Resources and Conservation Practices of the Agta of Lupigue, Ilagan City, Isabela, Philippines. *EDUCARE*, *8*(2), 115–128. https://doi.org/10.2121/EDU-IJES.V8I2.749

Gollock, M., Shiraishi, H., Carrizo, S., Crook, V., & Levy, E. (2018). *Status of non-CITES*

listed anguillid eels (Issue CITES AC30, p. Doc. 18.1 Annex 2). https://cites.org/sites/default/files/eng/com/ac/30/E-AC30-18-01-A2.pdf

Han, Y. S., Hung, C. L., Liao, Y. F., & Tzeng, W. N. (2010). Population genetic structure of the Japanese eel Anguilla japonica: Panmixia at spatial and temporal scales. *Marine Ecology Progress Series*, *401*, 221–232. https://doi.org/10.3354/meps08422

Han, Y. S., Lin, Y. F., Wu, C. R., Iizuka, Y., Castillo, T. R., Yambot, I. U., Mamalangkap, M. D., & Yambot, A. V. (2016). Biogeographic distribution of the eel Anguilla luzonensis: Dependence upon larval duration and oceanic currents. *Marine Ecology Progress Series*, *551*, 227–238. https://doi.org/10.3354/meps11728

Han, Y. S., Yambot, A. V, Zhang, H., & Hung, C. L. (2012). Sympatric spawning but allopatric distribution of anguilla japonica and anguilla marmorata: Temperature- and oceanic current-dependent sieving. *PLoS ONE*, *7*(6). https://doi.org/10.1371/journal.pone.0037484

Haralson, M. A., & Hassell, J. R. (1995). The extracellular matrix— an overview. In M. A. Haralson & J. R. Hassell (Eds.), *Extracellular Matrix: A Practical Approach* (pp. 1–30). IRL Press.

Hu, Z., Tong, Y., Manyande, A., & Du, H. (2020). Effective discrimination of flavours and tastes of Chinese traditional fish soups made from different regions of the silver carp using an electronic nose and electronic tongue. *Czech Journal of Food Sciences*, *38*(2), 84–93. https://doi.org/10.17221/103/2018-CJFS

Jafari, H., Lista, A., Siekapen, M. M., Ghaffari-Bohlouli, P., Nie, L., Alimoradi, H., & Shavandi, A. (2020). Fish collagen: Extraction, characterization, and applications for biomaterials engineering. In *Polymers* (Vol. 12, Issue 10, pp. 1–37). MDPI AG. https://doi.org/10.3390/polym12102230

Jamaluddin, Rusli, C., Yuyun, Y., & Widodo, A. (2019). Comparative Study of Fish Eel Amino Acid Profile (Anguilla marmorata (Q.) Gaimard) on Silver eel Phase from Palu River and Poso Lake. *Journal of Pharmacy and Nutrition Sciences*, *9*, 0–000.

Jamandre, B. W. D., Shen, K. N., Yambot, A. V., & Tzeng, W. N. (2007). Molecular phylogeny of philippine freshwater eels anguilla spp. (Actinopterygi: Anguilliformes: Anguillidae) inferred from mitochondrial DNA. *Raffles Bulletin of Zoology*, *2014*(14), 51–59.

Japan Wildlife Conservation Society (JWCS). (2017). *Eels in Japan; Habitat status, aquaculture and trade in Japan*. http://easec.info/EASEC_WEB/index_files/EASECdeclarations%28Final%29.pdf.

Jellyman, D. (2014). Freshwater Eels and People in New Zealand: A Love/Hate Relationship. In Katsumi Tsukamoto & M. Kuroki (Eds.), *Eels and Humans* (2nd ed., pp. 143–153). Springer Japan. https://doi.org/10.1007/978-4-431-54529-3_10

Jespersen, P. (1942) Indo-Pacific leptocephalids of the genus *Anguilla*. Dana Rep 22:1–128

Kita, T., Matsushige, K., Endo, S., Mochioka, N., & Tachihara, K. (2021). First Japanese records of Anguilla luzonensis (Osteichthyes: Anguilliformes: Anguillidae) glass eels from Okinawa-jima Island, Ryukyu Archipelago, Japan. *Species Diversity*, *26*(1), 31–36. https://doi.org/10.12782/specdiv.26.31

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Kuroki, M, Aoyama, J., Miller, M. J., Yoshinaga, T., Shinoda, A., Hagihara, S., & Tsukamoto, K. (2009). Sympatric spawning of Anguilla marmorata and Anguilla japonica in the western North Pacific Ocean. *Journal of Fish Biology*, *74*(9), 1853–1865. https://doi.org/10.1111/j.1095-8649.2009.02299.x

Kuroki, M., Aoyama, J., Miller, M. J., Wouthuyzen, S., Arai, T., & Tsukamoto, K. (2006). Contrasting patterns of growth and migration of tropical anguillid leptocephali in the western Pacific and Indonesian Seas . *Marine Ecology Progress Series*, *309*, 233–246. https://doi.org/10.3354/meps309233

Kuroki, M., Miller, M. J., Aoyama, J., Watanabe, S., Yoshinaga, T., & Tsukamoto, K. (2012). Offshore spawning for the newly discovered anguillid species Anguilla luzonensis (Teleostei: Anguillidae) in the western North Pacific. *Pacific Science*, *66*(4), 497–507. https://doi.org/10.2984/66.4.7

Kuroki, M., van Oijen, M. J. P., & Tsukamoto, K. (2014). Eels and the Japanese: An Inseparable, Long-Standing Relationship. In Katsumi Tsukamoto & M. Kuroki (Eds.), *Eels and Humans* (2nd ed., pp. 91–108). Springer Japan. https://doi.org/10.1007/978-4-431-54529-3_6

Le, Q. D., Nguyen, D. C., & Nguyen, D. T. (2012). Trace metal contents of tropical anguillid eels in Vietnam. *Coastal Marine Science*, *35*(1), 136–141.

Le, Q. D., Shirai, K., Nguyen, D. C., Miyazaki, N., & Arai, T. (2009). Heavy metals in a tropical eel Anguilla marmorata from the central part of vietnam. *Water, Air, and Soil Pollution*, *204*(1–4), 69–78. https://doi.org/10.1007/s11270-009-0027-7

Leander, N. J., Shen, K. N., Chen, R. T., & Tzeng, W. N. (2012). Species composition and seasonal occurrence of recruiting glass eels (Anguilla spp.) in the Hsiukuluan River, Eastern Taiwan. *Zoological Studies*, *51*(1), 59–71. http://zoolstud.sinica.edu.tw/Journals/51.1/59.pdf

Leander, N. J., Tzeng, W. N., Yeh, N. T., Shen, K. N., & Han, Y. S. (2013). Effects of metamorphosis timing and the larval growth rate on the latitudinal distribution of sympatric freshwater eels, anguilla japonica and *A. marmorata*, in the western North Pacific. *Zoological Studies*, *52*(1), 1–15. https://doi.org/10.1186/1810-522X-52-30

Lee, T. W. (2014). Eels and the Korean People. In Katsumi Tsukamoto & M. Kuroki (Eds.), *Eels and Humans* (2nd ed., pp. 109–116). Springer Japan. https://doi.org/10.1007/978-4-431-54529-3_7

Lin, Y. S., Poh, Y. P., Lin, S. M., & Tzeng, C. S. (2002). Molecular techniques to identify freshwater eels: RFLP analyses of PCR-amplified DNA fragments and allele-specific PCR from mitochondrial DNA. *Zoological Studies*, *41*(4), 421–430. http://www.sinica.edu.tw/zool/zoolstud/41.4/421.pdf

Luo, M.-Z., Guan, R.-Z., & Jin, H. (2015). Analysis on the Ratio of Flesh Content and the Nutritional Composition in the Muscle of Five Species of Eel. *ACTA HYDROBIOLOGICA SINICA, 2015, Vol. 39, Issue 4, Pages: 714-722*, *39*(4), 714–722. https://doi.org/10.7541/2015.94

Magulod Jr., G. (2018). Conservation beliefs and practices of indigenous people in Northwestern Philippines: implications for environmental promotion and education. *Journal of Biodiversity and Environmental Sciences (JBES)*, *12*(6), 186–194.

McCarthy, T. K. (2014). Eels and People in Ireland: From Mythology to International Eel Stock Conservation. In Katsumi Tsukamoto & M. Kuroki (Eds.), *Eels and Humans* (2nd ed., pp. 13–40). Springer Japan. https://doi.org/10.1007/978-4-431-54529-3_2

Merano, V. (n.d.-a). *Paksiw na Isda Recipe*. Retrieved June 3, 2021, from https://panlasangpinoy.com/*Paksiw*-na-isda-recipe/

Merano, V. (n.d.-b). *The Simplest Paksiw na Galunggong Recipe - Panlasang Pinoy*. Retrieved June 3, 2021, from https://panlasangpinoy.com/easy-simplest-*Paksiw*-na-galunggong-recipe/

Miller, M. J., Mochioka, N., Otake, T., & Tsukamoto, K. (2002). Evidence of a spawning area of Anguilla marmorata in the western North Pacific. *Marine Biology*, *140*(4), 809–814. https://doi.org/10.1007/s00227-001-0754-9

Miller, M., & Tsukamoto, K. (2004). *An introduction to leptocephali biology and identification* (pp. 1–96). Ocean Research Institute, The University of Tokyo. http://orton.catie.ac.cr/cgi-bin/wxis.exe/?IsisScript=LIBRI.xis&method=post&formato=2&cantidad=1&expresion=mfn=020376

Minegishi, Y., Aoyama, J., Inoue, J., Azanza, R., & Tsukamoto, K. (2009). Inter- specific and subspecific genetic divergences of freshwater eels, genus Anguilla including a recently described species, *A. luzonensis*, based on whole mitochondrial genome sequences. *Coastal Marine Science*, *33*(1), 64–77.

Minegishi, Y., Aoyama, J., Inoue, J. G., Miya, M., Nishida, M., & Tsukamoto, K. (2005). Molecular phylogeny and evolution of the freshwater eels genus Anguilla based on the whole mitochondrial genome sequences. *Molecular Phylogenetics and Evolution*, *34*(1), 134–146. https://doi.org/10.1016/j.ympev.2004.09.003

Moriarty, C. (2003). The Yellow Eel. In Katsumi Tsukamoto & M. Kuroki (Eds.), *Eel Biology* (2nd ed., pp. 89–105). Springer Japan. https://doi.org/10.1007/978-4-431-65907-5_7

Muthmainnah, D., Honda, S., Suryati, N. K., & Prisantoso, B. I. (2016). Understanding the Current Status of Anguillid Eel Fisheries in Southeast Asia. *Fish for the People*, *14*(3), 19–25. http://hdl.handle.net/20.500.12066/997

Nafsiyah, I., Nurilmala, M., & Abdullah, A. (2018). Nutrient Composition of Eel Anguilla bicolor bicolor and Anguilla marmorata. *Jurnal Pengolahan Hasil Perikanan Indonesia*, *21*(3), 504. https://doi.org/10.17844/jphpi.v21i3.24733

Nieves, P. M., & Nolial, J. C. C. (2019). Post-harvest handling practices for glass eel along rivers and tributaries in lagonoy gulf, Philippines. *AACL Bioflux*, *12*(5), 1662–1671. http://www.bioflux.com.ro/aacl

Nieves, P.M., Mendoza, A.B., Bradecina, R.G., Nolial, J.C., Celestial, N., Kubota, S., Canon, K.L. (2021) The Eel Fishery in Tributaries Along Lagonoy Gulf: Implications for Management and Conservation. BU R&D Journal: Bicol University, 24(1), 67-81. 10.47789/burdj.mbtcbbgs.20212401.11

Nutriasia. (2021). *Paksiw Na Isda*. Retrieved June 3, 2021, from https://nutriasia.com/recipes/*Paksiw*-na-isda/

Olaño, V., Lanzuela, N., & Paredes, K. (2018). Assessment of Fishery Resources in the Lagonoy Gulf, Philippines. *The Philippine Journal of Fisheries*, *25*(1), 62–76. https://doi.org/10.31398/tpjf/25.1.2017c0007

Özogul, Y., Özyurt, G., Özogul, F., Kuley, E., & Polat, A. (2005). Freshness assessment of European eel (Anguilla anguilla) by sensory, chemical and microbiological methods. *Food Chemistry*, *92*(4), 745–751. https://doi.org/10.1016/j.foodchem.2004.08.035

Philippine Statistics Authority (PSA) OpenSTAT (2020) Inland Municipal Fisheries: Volume of Production by Geolocation, Species, Year and Quarter. Retrieved June 26, 2020, from http://openstat.psa.gov.ph/PXWeb/pxweb/en/DB/DB__2E__FS/0052E4GVIP0.px/table/tableViewLayout1/?rxid=bdf9d8da-96f1-4100-ae09-18cb3eaeb313t

Polistico, E. (2017). *Philippine Food, Cooking, & Dining Dictionary*. books.google.com. https://books.google.com/books?hl=en&lr=&id=STSWDwAAQBAJ&oi=fnd&pg=PT2&dq=coconut+meat+grinder&ots=JAxiarjGkL&sig=WtSXw-8ojodxk2CtXYDXI2yP78Q

Qi, J., Xu, Y., Xie, X. fei, Zhang, W. wen, Wang, H. hu, Xu, X. lian, & Xiong, G. yuan. (2020). Gelatin enhances the flavor of chicken broth: A perspective on the ability of emulsions to bind volatile compounds. *Food Chemistry*, *333*(March), 127463. https://doi.org/10.1016/j.foodchem.2020.127463

Qixing, J., Zhengran, M., Shuoshuo, W., Yanshun, X., Fengyu, T., Xueqin, X., Peipei, Y., & Wenshui, X. (2014). Effect of temperature on protein compositional changes of big head carp (Aristichthys nobilis) muscle and exudates. *Food Science and Technology Research*, *20*(3), 655–661. https://doi.org/10.3136/fstr.20.655

Qu, T., & Lukas, R. (2003). The bifurcation of the North Equatorial Current in the Pacific. *Journal of Physical Oceanography*, *33*(1), 5–18. https://doi.org/10.1175/1520-0485(2003)033<0005:TBOTNE>2.0.CO;2

Randolph, M. (2018). *Why baby eels are one of Spain's most expensive foods*. BBC. https://www.bbc.com/travel/article/20180313-why-baby-eels-are-one-of-spains-most-expensive-foods

Righton, D., & Roberts, M. (2014). Eels and People in the United Kingdom. In Katsumi Tsukamoto & M. Kuroki (Eds.), *Eels and Humans* (2nd ed., pp. 1–12). Springer. https://doi.org/10.1007/978-4-431-54529-3_1

Rindom, S., Tomkiewicz, J., Munk, P., Aarestrup, K., Als, T. D., Pedersen, M. I., Graver, C., & Anderberg, C. (2014). Eels in Culture, Fisheries and Science in Denmark. In Katsumi Tsukamoto & M. Kuroki (Eds.), *Eels and Humans* (2nd ed., pp. 41–60). Springer Japan. https://doi.org/10.1007/978-4-431-54529-3_3

Ringuet, S., Muto, F., & Raymakers, C. (2002). Eels: Their Harvest and Trade in Europe and Asia. *TRAFFIC Bulletin*, *19*(2), 1–27.

Sato, K., Yoshinaka, R., Sato, M., & Shimizu, Y. (1986). Collagen Content in the Muscle of Fishes in Association with Their Swimming Movement and Meat Texture. *NIPPON SUISAN GAKKAISHI*, *52*(9), 1595–1600. https://doi.org/10.2331/suisan.52.1595

Sato, K., Sakuma, A., Ohtsuki, K., & Kawabata, M. (1994). Subunit Composition of Eel (*Anguilla japonica*) Type V Collagen: Evidence for Existence of a Novel Fourth α4(V) Chain. *Journal of Agricultural and Food Chemistry*, *42*(3), 675–678. https://doi.org/10.1

Shinoda, A., Yoshinaga, T., Aoyama, J., Tsuchida, G., Nakazato, S., Ishikawa, M., Matsugamoto, Y., Watanabe, S., Azanza, R., & Tsukamoto, K. (2015). Early life history of the Luzon mottled eel Anguilla luzonensis recruited to the Cagayan River, Luzon Island, the Philippines. *Coastal Marine Science*, *38*(1), 21–26. https://doi.org/10.15083/00040617

Shiraishi, H., & Crook, V. (2015). Eel market dynamics: An analysis of Anguilla production. In *TRAFFIC, Tokyo, Japan*.

Shirotori, F., Ishikawa, T., Tanaka, C., Aoyama, J., Shinoda, A., Yambot, A. V., & Yoshinaga, T. (2016). Species composition of anguillid glass eels recruited at southern Mindanao Island, the Philippines. *Fisheries Science*, *82*(6), 915–922. https://doi.org/10.1007/s12562-016-1030-8

Sikorski, Z. E., & Borderias, J. A. (1994). Collagen in the Muscles and Skin of Marine

Animals. In *Seafood Proteins* (pp. 58–70). Springer US. https://doi.org/10.1007/978-1-4615-7828-4_5

Sila, A., Martinez-Alvarez, O., Krichen, F., Gómez-Guillén, M. C., & Bougatef, A. (2017). Gelatin prepared from European eel (Anguilla anguilla) skin: Physicochemical, textural, viscoelastic and surface properties. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *529*(March), 643–650. https://doi.org/10.1016/j.colsurfa.2017.06.032

Soliman, V. S. (2013). Managing at the ‘ Root ’ of Kuroshio. *黒潮圏科学（Kuroshio Science）*, *7*(1), 31–39. http://www.bas.gov.ph

Song, D.-J., Ha, J.-H., & Lee, E.-H. (1982). Studies on Histological Changes in Sea Foods during Processing and Storage 1. Changes in Muscular Tissue and Fat Migration of Eel, Anguilla japonica, during Drying. *Korean Journal of Fisheries and Aquatic Sciences*, *15*(2), 137–146.

Song, D.-J., & Lee, E.-H. (1982). Studies on Histological Changes in Marine Foods during Processing and Storage 2. Changes in muscular tissue of the eel, Anguilla japonica, by freezing storage. *Korean Journal of Fisheries and Aquatic Sciences*, *15*(3), 199–206.

Southeast Asian Fisheries Development Center (SEAFDEC). (2019). *REPORT on the JAIF Project “Enhancing Sustainable Utilization and Management Scheme of Tropical Anguillid Eel Resources in Southeast Asia” Southeast Asian Fisheries Development Center*.

Sugeha, H. Y., & Arai, T. (2010). Contrasting Morphology, Genetic, and Recruitment Season of. *ILMU KELAUTAN*, *1*, 1–19.

Sugeha, H. Y., Suharti, S. R., Wouthuyzen, S., & Sumadhiharga, K. (2008). Biodiversity, Distribution, and Abundance of the Tropical Anguillid Eels in the Indonesian Waters. *Marine Research in Indonesia*, *33*(2), 129–137. https://doi.org/10.14203/mri.v33i2.486

Suryati, N. K., Pamungkas, Y. P., & Muthmainnah, D. (2019). Addressing the Issues and Concerns on Anguillid Eel Fisheries in Southeast Asia Current Status of the Tropical Anguillid. *Fish for the People*, *17*(1), 19–24. http://repository.seafdec.org/bitstream/handle/20.500.12066/4811/issues_and_concerns_anguillid_eel.pdf?sequence=1&isAllowed=y%0Ahttp://repository.seafdec.org/handle/20.500.12066/4811

Tabeta, O., Takai, T., & Matsui, I. (1976). The sectional counts of vertebrae in the anguillid elvers. *Japan. J. Ichthyol. 2*, *22*, 195–200.

Tabeta, O, Tanimoto, T., Takai, T., Matsui, I., & Imamura, T. (1976). Seasonal Occurrence of Anguillid Elvers in Cagayan River, Luzon Island, the Philippines. *NIPPON SUISAN GAKKAISHI*, *42*(4), 421–426. https://doi.org/10.2331/suisan.42.421

Tabeta, Osame, & Mochioka, N. (2003). The Glass Eel. In K. Aida, K. Tsukamoto, & K. Yamauchi (Eds.), *Eel Biology* (pp. 75–87). Springer Japan. https://doi.org/10.1007/978-4-431-65907-5_6

Tagliavini, J., Gandolfi, G., Deiana, A. M., & Salvadori, S. (1996). Phylogenetic relationships among two atlantic and three indo-pacific anguilla species (osteichthyes, anguillidae). *Italian Journal of Zoology*, *63*(3), 271–276. https://doi.org/10.1080/11250009609356144

Tahergorabi, R., Hosseini, S. V., & Jaczynski, J. (2011). Seafood proteins. In *Handbook of Food Proteins* (pp. 116–149). Elsevier. https://doi.org/10.1533/9780857093639.116

Teng, H. Y., Lin, Y. S., & Tzeng, C. S. (2009). A new Anguilla species and a reanalysis of the phylogeny of freshwater eels. *Zoological Studies*, *48*(6), 808–822. http://zoolstud.sinica.edu.tw/Journals/48.6/808.pdf

Tesch, F.-W., & Greenwood, P. H. (1977). Eel Species, their Developmental Stages and their Distribution. In P. H. Greenwood (Ed.), *The Eel* (pp. 81–132). Springer Netherlands. https://doi.org/10.1007/978-94-009-5761-9_2

The Freeman (2016) Inun-unan Isda. Retrieved June 5, 2020, from https://www.philstar.com/the-freeman/cebu-lifestyle/2016/09/02/1619672/inun-unan-isda

Tiangson-Bayaga, C. L. P., & Deveza, G. F. (2005). Milkfish (Chanos chanos Forskaal) consumption in the Philippines and the docosahexaenoic acid level of the cooked fish. *Food Science and Technology Research*, *11*(1), 127–133. https://doi.org/10.3136/fstr.11.127

Tornberg, E. (2005). Effects of heat on meat proteins - Implications on structure and quality of meat products. *Meat Science*, *70*(3 SPEC. ISS.), 493–508. https://doi.org/10.1016/j.meatsci.2004.11.021

Tsukamoto, K., Aoyama, J., & Miller, M. J. (2002). Migration, speciation, and the evolution of diadromy in anguillid eels. *Canadian Journal of Fisheries and Aquatic Sciences*, *59*(12), 1989–1998. https://doi.org/10.1139/f02-165

Tsukamoto, Katsumi. (1992). Discovery of the spawning area for Japanese eel. *Nature*, *356*(6372), 789–791. https://doi.org/10.1038/356789a0

Tzeng, W.-N. (2014). Freshwater Eels and Humans in Taiwan. In Katsumi Tsukamoto & M. Kuroki (Eds.), *Eels and Humans* (2nd ed., pp. 129–142). Springer Japan. https://doi.org/10.1007/978-4-431-54529-3_9

United Nations, & United Nations (UN). (2016). *World Wildlife Crime Report Trafficking in protected species*. www.unodc.org

United Nations Commodity Trade (UN Comtrade) Statistics Database. (2020). Live eels export of the Philippines to the World. Retrieved July 29, 2020, from

https://comtrade.un.org/db/dqBasicQueryResults.aspx?cc=030192&px=HS&r=608&y=2019,2018,2017,2016,2015,2014,2013,2012,2011,2010,2009,2008,2007,2006,2005,2004,2003,2002,2001,2000&p=0&rg=2&so=8.

van Herwaarden, H. (2003). *Eel culture in the Philippines*. http://home.planet.nl/~herwa073/aquacrops/eels/eels.html

Venugopal, V., & Shahidi, F. (1996). Structure and composition of fish muscle. *Food Reviews International*, *12*(2), 175–197. https://doi.org/10.1080/87559129609541074

Watanabe, S. (2003). Taxonomy of the Freshwater Eels, Genus Anguilla Schrank, 1798. In Katsumi Aida, K. Tsukamoto, & K. Yamauchi (Eds.), *Eel Biology* (pp. 3–18). Springer, Dordrecht. https://doi.org/10.1007/978-4-431-65907-5_1

Watanabe, S., Aoyama, J., Hagihara, S., Ai, B., Azanza, R. V., & Tsukamoto, K. (2013). Anguilla huangi Teng, Lin, and Tzeng, 2009, is a junior synonym of Anguilla luzonensis Watanabe, Aoyama, and Tsukamoto, 2009. *Fisheries Science*, *79*(3), 375–383. https://doi.org/10.1007/s12562-013-0620-y

Watanabe, S., Aoyama, J., Nishida, M., & Tsukamoto, K. (2005). A molecular genetic evaluation of the taxonomy of eels of the genus Anguilla (Pisces: Anguilliformes). *Bulletin of Marine Science*, *76*(3), 675–690.

Watanabe, S., Aoyama, J., & Tsukamoto, K. (2004). Reexamination of Ege's (1939) use of taxonomic characters of the genus Anguilla. *Bulletin of Marine Science*, *74*(2), 337–351.

Watanabe, S., Aoyama, J., & Tsukamoto, K. (2009). A new species of freshwater eel Anguilla luzonensis (Teleostei: Anguillidae) from Luzon Island of the Philippines. *Fisheries Science*, *75*(2), 387–392. https://doi.org/10.1007/s12562-009-0087-z

Wattanachant, S., Benjakul, S., & Ledward, D. A. (2005). Effect of heat treatment on changes in texture, structure and properties of Thai indigenous chicken muscle. *Food Chemistry*, *93*(2), 337–348. https://doi.org/10.1016/j.foodchem.2004.09.032

Wouthuyzen, S., Aoyama, J., Sugeha, H. Y., Miller, M. J., Kuroki, M., Minegishi, Y., Suharti, S. R., & Tsukamoto, K. (2009). Seasonality of spawning by tropical anguillid eels around Sulawesi Island, Indonesia. *Naturwissenschaften*, *96*(1), 153–158. https://doi.org/10.1007/s00114-008-0457-x

Yang T., S., & Lee H., E. (1980). Taste Compounds of Fresh-Water Fishes. 3. Taste Compounds of Korean Snakehead Meat. *BULL. KOREAN FISH. SOC.*, *13*(3), 115–119.

Yap, W. G., Villaluz, A. C., Soriano, M. G. G., & Santos, M. N. (2007). Milkfish Production and Processing Technologies in the Philippines. *Milkfish Project Publication Series No. 2*, *2*, 96. http://www.worldfishcenter.org/resource_centre/WF_783.pdf

Yoshinaga, T., Aoyama, J., Shinoda, A., Watanabe, S., Azanza, R. V., & Tsukamoto, K. (2014). Occurrence and biological characteristics of glass eels of the Japanese eel Anguilla Japonica at the Cagayan river of Luzon island, Philippines in 2009. *Zoological Studies*, *53*(1), 1–6. https://doi.org/10.1186/1810-522X-53-13

Yoshinaka, R., Sato, K., Anbe, H., Sato, M., & Shimizu, Y. (1988). Distribution of collagen in body muscle of fishes with different swimming modes. *Comparative Biochemistry and Physiology -- Part B: Biochemistry And*, *89*(1), 147–151. https://doi.org/10.1016/0305-0491(88)90276-3

Zhang, J., Yao, Y., Ye, X., Fang, Z., Chen, J., Wu, D., Liu, D., & Hu, Y. (2013a). Effect of cooking temperatures on protein hydrolysates and sensory quality in crucian carp (Carassius auratus) soup. *Journal of Food Science and Technology*, *50*(3), 542–548. https://doi.org/10.1007/s13197-011-0376-2

Zhang, J., Yao, Y., Ye, X., Fang, Z., Chen, J., Wu, D., Liu, D., & Hu, Y. (2013b). Effect of cooking temperatures on protein hydrolysates and sensory quality in crucian carp (Carassius auratus) soup. *Journal of Food Science and Technology*, *50*(3), 542–548. https://doi.org/10.1007/s13197-011-0376-2

Zhiyong, M. (1998). The Comparative Study of Biochemical Compositions of Muscle of Anguilla Marmorata and Anguilla Japonica--《JOURNAL OF JIMEI UNIVERSITY NATURAL SCIENCE》1998年03期. *Journal of Jimei University Natural Science*, *3*(3), 132–135. https://en.cnki.com.cn/Article_en/CJFDTotal-JMXZ199803025.htm

Appendix

Interview Questions

A. Respondents Profile

Name: _____ Age:

Address: _____ Years of stay: _____

Occupation/Source of living: _____ No. of family members: _____

Estimated Annual Income: _____

B. Collection/ Harvest

1. What species of eels are usually caught in the area?
2. What stages of eel do you usually catch?
3. How is it being collected? (tools/method)
4. What are the sizes of eels usually collected (juvenile, elvers, yellow eels)?
5. What season do you usually catch eels (juvenile, elvers, yellow eels)?
6. At which part of the river do you usually catch eels?
7. Which is the best season to collect eels?
8. When is the spawning season of eels?
9. Do you need permits in collecting eels?
10. Who issues permit for eel collection?

C. Cooking

1. Are you consuming the eels you have collected?
2. How many times do you consume it in a week?
   ___ once ___ twice ___thrice ___4x ___ none at all
3. How do you cook it/prepare for consumption?
4. Do you process it like drying and etc? How?

D.

1. Are you selling the eels you have collected?
2. To whom are you selling the collected eels?
3. How much are you being paid for catching and selling approximately 1 kg of eel?
4. How do you transport the collected eels to your client?