Cytochrome oxidase-I sequence based studies of commercially available *Pangasius hypophthalmus* in Italy

Federica Bellagamba, Dinesh Velayutham, Maria Cristina Cozzi, Fabio Caprino, Mauro Vasoni, Maria Letizia Busetto, Alessandro Bagnato, Vittorio Maria Moretti

Dipartimento di Scienze Veterinarie per la Salute, la Produzione Animale e la Sicurezza Alimentare, University of Milan, Italy

**Abstract**

*Pangasius hypophthalmus* is one of the fish consumed in the Italian diet. It is farmed and imported from Mekong delta region of Vietnam. Among several types of Pangasius, *Tra* (*Pangasius hypophthalmus*) is permitted for sales by the European Union. Since these fish species are often allegedly substituted with other morphologically similar fish due to commercial benefits, authentication of the products in the international markets become often necessary to prevent fraud and safety issues. In addition, this fish is imported as fillets without skin and bone, thus leaving the consumer’s at the risk of buying a substandard nutritional food. In this article we present the molecular approach we developed to identify *Pangasius hypophthalmus* from other closely related species based on cytochrome oxidase-I (COI) mitochondrial barcoding gene and further described the variants in the studied population genetic of this species. Fifty-one samples of *Pangasius hypophthalmus* fillets labelled as *Pangasio* were obtained from various markets around Milan and their COI mitochondrial barcoding gene was sequenced and studied in our bioinformatics pipeline. All samples were successfully amplified and Basic Local Alignment Search Tool results of the amplified region confirmed that all sequences analysed belonged to *Pangasius hypophthalmus*. Based on the variations in their barcoding region single nucleotide polymorphisms were identified and delineative statistics was calculated on the sequences. Although *Pangasius hypophthalmus* is considered as a monophyle, seven polymorphisms were identified. The neighbour-joining tree and the Median-joining network of haplotypes showed for all the identified haplotypes a unique cluster, with the exception of one sample.

**Introduction**

*Pangasius hypophthalmus*, also called as *Sutchi catfish or Tra*, is one of the commercially important and economically viable fish. Native to tropical Asian countries, *Tra* farming has excellent growth records in the last decade, particularly in Vietnam, which is also the leading exporter of this fish. *Tra* and *Basa* (*Pangasius bocourti*), another variant of this fish helps in improving the economic situation and provides more employment opportunities for the people of Vietnam. As quoted by FAO (2005), the total fish production from both capture fisheries and fish culture in Vietnam was about 2,226,000 tons in 2001 and the production quantity is doubled in 2011. In 2013 about 20 percentage of Pangasius produced in Vietnam were imported in European Union (EU), for a value of 1.3 billion US dollars (Pangasius-Vietnam, 2014). Now due to high export of these fishes, there is the problem of level of authenticity of the products into the international markets. The EU is keen in overcoming economic fraud and food safety issues, which implies species authorization, not only for health concern but also for trade and tariff restrictions. So all aquatic species imported, harvested, processed and sold in Italy are subjected to EU regulations (104/2000/EU, 2063/2001/EU; European Commission, 2000, 2001), for their safety and label. Fish processing often removes or damages the diagnostic characters crucial for species identification by conventional taxonomic methods. *Tra* is one of the important nutritional (Orban et al., 2008; Busetto et al., 2011) and economically significant species in Italy as fish of value around 26.5 US dollars were imported in the first eleven months of 2012 (VASEP, 2014). *Tra* has been imported from Vietnam as skinless fillets to Italy and sold as *Pangasio*, the fillets vary in color from pink to dark red. One of the challenges for consumers and regulators in markets is the detection of species substitution in whole or partially with that of low quality species or with potential toxin species. Species authentication of *Tra* is a challenge for authorities, as these species are imported as fillets. Pangasius has been very less studied for their nutritional characteristics, species authentication with molecular methodology prior to study the species is mandatory. In the last decades molecular analysis has been an alternative to taxonomic identification (Wolf et al., 1999; Doodley et al., 2005). Advances in nucleotide sequencing techniques have established affordable, accurate and an easy approach for species identification by sequencing a short unique molecule of the genome (Pepe et al., 2005). DNA barcoding was accepted as a method to identify species, by means of the amplification and sequencing of a short universal molecular tag of approximately 650 base pairs of the mitochondrial cytochrome oxidase subunit I (COI) gene. Consortium for the barcode of life (CBOL) states COI as barcode gene to identify species. DNA-barcoding has found a valid employment in various biological studies (Wong and Hanner, 2008). The Barcode of life data Systems (BOLD) adopted DNA-barcoding technology to produce extensive libraries tend to provide the most valid tool for species identification for all fish (Ward et al., 2005). One of the reasons for COI being selected as the barcode is because it is a mitochondrial gene. In animal species, mitochondrial DNA exists as a circular molecule containing 13 protein coding regions, ribosomal genes, a non-protein coding region and several tRNA (Jameson et al., 2003). Multiple existence of such circular molecule in mitochondria and multiple mitochondria in a cell facilitate abundance of DNA sample, even at less availability of tissue samples. The rate of mutation in DNA segments is inversely related to their length (Drake et al., 1982), hence for the barcode analysis nuclear DNA requires a larger segment when compared with the mtDNA. With short sequences and reliable variability among each species, **Corresponding author:** Dr. Federica Bellagamba, Dipartimento di Scienze veterinarie per la salute, la produzione animale e la sicurezza alimentare (VESPA), Università degli Studi di Milano, Via Trentacoste 2, 20139 Milano, Italy. Tel. +39.02.50315759 - Fax: +39.02.50315746. E-mail: federica.bellagamba@unimi.it

Key words: Pangasius; Species authentication; COI gene.

Received for publication: 27 February 2015. Accepted for publication: 30 March 2015.

This work is licensed under a Creative Commons Attribution NonCommercial 3.0 License (CC BY-NC 3.0).

©Copyright F. Bellagamba et al., 2015

Licensee PAGEPress, Italy

Italian Journal of Animal Science 2015; volume 14:3928
doi:10.4081/ijas.2015.3928
mtDNA is favoured for species identification. According to Blaxter (2004) intraspecific variation between species for COI is less than 10%. Several authors have demonstrated that COI is highly appropriate for differentiating between closely related species across diverse phyla and for identifying animals, including fish, (Hebert et al., 2003a, 2003b; Ward et al., 2005; Wong and Hanner, 2008; Azlina et al., 2013). Other than identifying a species, the knowledge of the structure of each stock is very important for a better commercial exploitation. In fish, the population genetic structure subjects to change over time influenced by biological and physical factors. Based on the physical, chemical or biological environment fish can respond in three different ways by migrating to a favourable place, phenotypically adapting to the conditions or genotypically adapting to the altered environment (Reusch and Wood, 2007; Gineapp et al., 2008). Population study is an important tool in fisheries management and conservation. Pangasius Aquaculture Dialogue (PAD, 2007) convened by World 100 Wildlife Fund (WWF) addressed issues of genetic integrity of local population, potential threat of Genetically Modified Organism (GMOs) and hybridization with closely related species. With lot of Pangasius hybridization experiments being performed within Pangasiidae family and other families (Tarnchalanukit, 1986) as farming practice by farmers for better production, the genomic pattern of Pangasius is at high risk. A similar molecular barcoding study reveals 28 percent of substitution in fish (Filonzi et al., 2010) and 25 percent of substitution in seafood (Wong and Hanner 2008), which is alleged a serious fraud under economic and nutritional concerns. The current study was aimed to i) develop a molecular methodology to identify the species sold in Italy labeled as Pangasio; ii) apply the methodology to authenticate the species before nutritional characterization and iii) identify the common polymorphism inside the barcoding gene of Pangasius hypophthalmus.

Materials and methods

Fifty-one samples of Pangasius hypophthalmus fillets named in their product label as ‘Pangasio’ was obtained from various markets around Milan for a period of one year 2008 - 2009. The samples were registered as Panga 1, Panga 2, up to Panga 51 and the fillet samples were stored at -20°C until extraction of DNA. Total DNA was extracted from 5 mg of homogenized tissue samples, by 1 ml of lysis buffer [10mM Tris Hcl (pH 7.5), 150m NaCl, 2mM EDTA, 1 % sodium dodecyl sulfate] and 40 uL of proteinase K (10mg/mL)]. The lysate was treated in Wizard mini column (Promega, Madison, WI, USA), and the DNA was isolated according to the manufacture’s instruction. Primers were designed taking into account of four species Pconchophilus, P. gigas, P. hypophthalmus and P. bocourti; P.gigas and P. hypophthalmus shares a subfamily Pangasianodon; P. conchophilus, P. bocourti and P. hypophthalmus are commercially related and are often misunderstood for the other. The amplification of mitochondrial COI gene was carried out in a 50 µL final volume with primers (Invitrogen-Italy): Fwd 5’- ATGCCCCATGTAATAATTTTC-3’ and Rev 5’-GTTAGrATATwGTAATGCCrGC-3’. These primers were not specific for Pangasius hypophthalmus, but a common primer for four closely related species, (Pangasius hypophthalmus, Pangasius gigas, Pangasius bocourti and Pangasius conchophilus) which are considered as a cause for economic deception. The forward primer was conserved in all the referred four species, instead the reverse primer had three variable regions, which were replaced by degenerative nucleotides (r=C and T; w=A and T). The primers cover 454 base pairs (bp) of the aligned sequences from the four species considered.

By this way even if the commercially available samples were any of the above species, the specie could be identified after sequencing. PCR amplification was performed in Gene Amp PCR systems 2400 (Perkin Elmer), cycling conditions were as follows: 40 s at 98°C; 25 cycles of 94°C for 30 s and 50°C, 72°C 30s; and a final extension of 5 min at 68°C. The amplification was also separated in a 2% agarose gel (SeaKem, GTG, Agarose) for band at the size of 450 bp and sequenced by Sanger sequencing method. Two samples (Panga 10 and Panga 22) reported poor sequencing and were not considered for analysis. The sequences obtained were edited manually and multiple sequence alignment was performed using CLUSTAL X (Thompson et al., 1997) and MEGA (Tamura et al., 2007). The aligned barcode sequences were further blasted in National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) to identify their species. Further haplotype identification was performed in DnaSP v5 and their genetic diversity within the species population was calculated, the population structure of fish samples was studied using MEGA. Delineate statistics such as nucleotide composition, variable sites, parsimony informative sites, average number of pairwise nucleotide difference and disparity index were calculated using MEGA. The average nucleotide substitution pattern between sequences was estimated through Maximum likelihood method, Codon positions included were 1st+2nd+3rd+ Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete-deletion option). There were a total of 379 positions in the final dataset. All calculations were conducted in MEGA, which provided the probability of substitution from one base to another and also the average nucleotide pairs. A median-joining network (Bandelt et al., 1999) relating the haplotypes identified and the reference sequence was constructed using the software Network 4.6.1.1. (www.fluxus-engineering.com).

Results and discussion

All the samples used for study were successfully amplified (Figure 1) and BLAST results of the amplified barcoding region confirmed that all the sequences analyzed were of Pangasius hypophthalmus (Table 1). Among the 49 analyzed COI gene sequences, seven haplotypes were found and the sequences were published in literature with accession numbers GU32234B - GU322358 (Velayutham et al., 2010). Singleton

![Figure 1. Agarose gel electrophoresis of amplified Cytochrome oxidase-I gene subunit, where M is 100 bp ladder, numbers 36-43 are panga samples, and B is blank (without template DNA).](image-url)
variable site positions in the aligned sequences were 5522, 5593, 5798, 5806 and 5886; similarly, parsimony informative sites were 5837 and 5888. Polymorphism at site 5837 was replicated in 11 samples and polymorphism site 5888 was replicated in 9 samples (Table 2). Haplotype frequencies were reported in Table 2, the haplotype H1 was found in the 63% of samples, whereas the lowest frequency was found for haplotype H2, H6 and H7 (2%). The haplotype diversity (HD) calculated with DnaSP v5 was 0.565 with variance of haplotype diversity 0.00551, the overall nucleotide diversity is also high (Pi = 0.00225). Among the sequences A/T base content was higher than G/C content, mean A = 25.1%, T = 33.3 %, G = 16.9 % and C = 24.8%. The results of nucleotide composition confirmed that the guanine was the lower (mean = 16.9 %) than other bases. Previously results (Azilina et al., 2013) obtained from COI gene sequences of different pangasiidae species reported the same conclusion with a G mean value of 18.2 %. The substitution pattern of nucleotide between sequences (Tables 3 and 4) showed a predominant transitional substitution (si), i.e. purine and pyrimidine were replaced by their same group, and the transversional substitution (sv) pattern was not observed. Thus in the sequences the purine and pyrimidine groups are not substituting each other, when there is a substitution of base. The transition/transversion rate ratios was k1=1.442 (purines) and k2=1.2069 (pyrimidines). The overall transition/transversion bias is R = 4.341.

The statistical null hypothesis for substitution pattern is that when the transitional and transversional substitution are similar, the Ratio R (si/sv) is 0.5, as there are twice as possibility for transversion substitution than transition substitution. Whereas the analysed samples had a predominant transition substitution rejecting the null hypothesis with an R value 3.5. These results were further supported by the Maximum likelihood estimate of the pattern of nucleotide substitution (Table 5), which reveals the transversional substitution values were considerably low. Considering the transitional substitution, to understand if these substitutions have caused any significant advantage of the population expansion, we studied the population

Table 1. National Center for Biotechnology Information Basic Local Alignment Search Tool results of amplified 433 bp sample sequence of *Cytochrome oxidase-I* gene.

| Accession   | Description       | Max score | Query coverage, % | E value | Identity, % |
|-------------|-------------------|-----------|-------------------|---------|--------------|
| EU752151.1  | *Phypothalmus*    | 689       | 100               | 0.0     | 99           |
| EF909427.1  | *Phypothalmus*    | 689       | 100               | 0.0     | 99           |
| EU595057.1  | *T.sinensis*      | 689       | 100               | 0.0     | 99           |
| AY76297.1   | *P.gigas*         | 468       | 100               | 1e-128  | 89           |
| EU671046.1  | *P.pangasius*     | 418       | 100               | 1e-113  | 86           |

Table 2. Haplotypes identified, number of samples per haplotype, haplotype frequencies and nucleotide substitution sites referred to the reference sequence for *Pangasius hypophthalmus* (EF609427.1).

| Haplotype | Number samples/haplotype | Haplotype frequencies | Nucleotide substitution sites |
|-----------|--------------------------|-----------------------|------------------------------|
|           |                          |                       | 5522 | 5593 | 5798 | 5806 | 5837 | 5886 | 5888 |
| H1        | 31                       | 0.63                  | -    | -    | -    | -    | -    | -    | -    |
| H2        | 1                        | 0.02                  | A    | -    | -    | -    | -    | -    | -    |
| H3        | 8                        | 0.16                  | -    | -    | -    | C    | -    | -    | -    |
| H4        | 4                        | 0.08                  | -    | -    | -    | -    | -    | T    | -    |
| H5        | 3                        | 0.06                  | -    | -    | -    | C    | -    | -    | T    |
| H6        | 1                        | 0.02                  | -    | T    | -    | -    | -    | T    | -    |
| H7        | 1                        | 0.02                  | -    | -    | T    | T    | -    | T    | T    |

Table 3. Average frequency of nucleotide pairs between sequences.

| Nucleotide pairs | TT | TC | TA | TG | CC | CA | CG | AA | AG | GG |
|------------------|----|----|----|----|----|----|----|----|----|----|
| Average          | 131| 1   | 0  | 0  | 95 | 0  | 0  | 100| 0  | 66 |
| 1st position     | 33 | 1   | 0  | 0  | 28 | 0  | 0  | 38 | 0  | 32 |
| 2nd position     | 47 | 0   | 0  | 0  | 41 | 0  | 0  | 18 | 0  | 24 |
| 3rd position     | 51 | 0   | 0  | 0  | 26 | 0  | 0  | 43 | 0  | 10 |

Figure 2. Phylogeny bootstrap analysis of neighbour joining tree for the 49 Pangasio *Cytochrome oxidase-I* sequences at bootstrap cut off value 50.
structure of these sequences by Tajima’s D. With respect to this substitution pattern and the observed negative D value (-1.2043) for the test, indicates a growing population size. The probability of the D values were not significant at < 0.01 level indicating a neutral population size. These results were further strongly confirmed by Fu and Li’s D* (-2.6516) and F* (-2.5708) test values. We further examined the probable mutation steps between haplotype by constructing the minimum haplotype spanning tree using Arlequin v3.5 software (Excoffier et al., 1992). Results of spanning tree shows two clear lineages for a bootstrap significant value of 50, with one sample being the outsider (Figure 2). The neighbour-joining tree of haplotypes (Saitou and Nei, 1987) based on the Kimura two parameter model (Kimura, 1980) showed that all the haplotypes but H7, were grouped in a cluster (Figure 3). The bootstrap values were lower than 50. The same result was found with the Median-joining network relating the 7 haplotypes identified on the Pangasio COI sequences referred to the sequence EF609427.1. Haplotype 7 clearly differs from the others by the singleton variable sites 5798, 5806 and 5886. The parsimony informative site 5837 identified the difference between haplotypes H3-H2 and H1-H4, whereas the parsimony informative site 5888 identified those between H1-H3 and H2-H4 (Figure 4).

Conclusions

In this study we report the polymorphism of barcoding gene in commercially available Pangasius hypophthalmus. Barcoding region COI confirms that all the fish purchased through this method sold as Pangasio in Italian markets were Tra (Pangasius hypophthalmus). This information could be very useful for the consumers, as these fish have no morphological tools for their identification when sold in the market as fillets without skin. In spite the contradictory appearance of the fillets with varying shades from pale to dark pink, we conclude that these observations could be due to the mode of slaughtering, where improper bleeding can cause various colours. Although Pangasius hypophthalmus is considered a monophyly the identified haplotype sequences add additional information to the literature of COI barcode region of this species. As said by Hebert et al. (2003a, 2003b) COI database collection serve as the basis for a global identification system (Genotyping by Sequencing, GBS) for animals, which was aimed to cover the comprehensive taxonomy coverage of COI gene alone. In future this micro genomic identification method of GBS could be revolutionary concept for a taxonomist in overcoming morphological identification bias and in studying population phylogenetic structure and molecular evidence of families.

Figure 3. Neighbour-joining tree relating the 7 haplotypes identified on the Pangasio Cytochrome oxidase-I sequences referred to the sequence for Pangasius hypophthalmus (GeneBank accession number EF609427.1).

Figure 4. Median-joining network tree of the 7 haplotypes identified on the Pangasio Cytochrome oxidase-I sequences referred to the sequence for Pangasius hypophthalmus (GeneBank accession number EF609427.1). The numbers between the haplotype nodes refer to the positions of nucleotide mutations.

Table 4. Average substitution pattern of nucleotide between sequences.

| Substitution pattern | si 1 | sv 2 | R 3 |
|----------------------|------|------|-----|
| Average              | 1    | 0    | 3.5 |
| 1st position (1-131) | 1    | 0    | 17.2|
| 2nd position (132-262)| 0    | 0    | 0.0 |
| 3rd position (263-392)| 0    | 0    | 0.3 |

si, transitional substitution; sv, transversional substitution; R, si/sv ratio.

Table 5. Maximum likelihood estimate of the pattern of nucleotide substitution.

| Nucleotide | A  | T  | C  | G  |
|------------|----|----|----|----|
| A          | -  | 3.46| 2.58| 2.53|
| T          | 2.61| -  | 31.15| 1.75|
| C          | 2.61| 41.75| -  | 1.75|
| G          | 3.76| 3.46| 2.58| -  |
References

Azlina, Z.N., Daud, S.K., Siraj, S.S., Aliabadian, M., Moghaddam, F.Y., 2013. Molecular phyloge- ny of Malaysian Pangasiid based on cytochrome C oxidase I (COI) DNA sequences. Iran. J. Anim. Biosystematics 9:17-28.

Bandelt, H.-J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16:37-48.

Blaxter, M.L., 2004. The promise of a DNA taxon- omy. Phil. Trans. R. Soc. Lond. B 359:669.

Bussetto, M.L., Caprino, F., Velayutham, D., Vasconi, M., Bellagamba, F., Moretti, M.V., 2011. Analysis of the nutritional properties of sutchi catfish (Pangasius hypophthalmus) fillets available on the Italian mar- ket. Ind. Aliment. 2011:1-6.

Doodley, J.J., Sage, H.D., Clarke, M.A., Brown, H.M., Garret, S.D., 2005. Fish species identifi- cation using PCR-RFLP analysis and lab-on-a-chip capillary electrophoresis: application to detect white fish species in food products an interlaboratory study. J. Agric. Food Chem. 4:3348-3357.

Drake, J.W., Charlesworth, B., Charlesworth, D., Crow, J.F., 1982. Rates of spontaneous mutation. Genetics 148:1667-1686.

European Commission, 2000. Council Regulation of 17 December 1999 on the common organisation of the market in fishery and aquaculture products, 104/2000/EC. In: Official Journal, L 17/22, 21-01-2000.

European Commission, 2001. Commission Regulation of 22 October 2001 laying down detailed rules for the application of Council Regulation (EC) No 104/2000 as regards informing consumers about fishery and aquaculture products, 2065/2001/EC. In: Official Journal, L 278, 22-10-2001.

Excoffier, L., Smouse, PE., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplo- types: application to human mitochondrial DNA restriction data. Genetics 131:479-491.

FAO, 2005. Vietnam national aquaculture sec- tor overview. Available from: http://www.fao.org/fishery/countrysector/n aos_vietnam/en

Filozzi, L., Chiesa, S., Vaghi, M., Marzano, N.F., 2010. Molecular barcoding reveals msla- belling of commercial fish products in Italy. Food Res. Int. 43:1383-1388.

Gineapp, P., Teplitsky, C., Alho, J.S., Mills, J.A., Merila, J., 2008. Climate change and evo- lution: disentangling environmental and genetic. Mol. Ecol. 1:167-168.

Hebert, P.D., Cywinski, A., Ball, S.L., deWaard, J.R., 2003a. Biological identifications through DNA barcodes. Proc. Biol. Sci. 270:313-321.

Hebert, P.D., Ratnasingham, S., De Waard, J.R., 2003b. Barcoding animal life: cytochrome c oxidase subunit I divergences among closely related species. Proc. Biol. Sci. 270:596-99.

Jameson, D., Gibson, A.P., Hudelot, C., Higgs, P.G., 2003. OGRE: a relational database for comparative analysis of mitochondrial genomes. Nucleic Acids Res. 31:202-206.

Kimura, M., 1980. A simple method for esti- mating evolutionary rate of base substitu- tions through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111-120.

Orban, E., Neigvato, Lena, G.D., Masci, M., Casini, I., Gambelli, L., Caproni, R., 2008. New trends in the seafood market. Sutchi catfish (Pangasius hypophthalmus) fillets from Vietnam: nutritional quality and safety aspects. Food Chem. 110:383-389.

Pangasiis-Vietnam, 2014. Available from: http://www.pangasius-vietnam.com

PAD, 2007. Development standards for sustain- able Pangasius farming. Proc. 1st meeting of the Pangasius Aquaculture Dialogue, Vietnam.

Pepe, T., Trota, M., Di Marco, I., Anastasio, A., Bautista, J.M., Cortesi, M.L., 2005. Mitochondrial cytochrome b DNA sequence variations: an approach to fish species identification in processed fish products. J. Food Prot. 68:421-425.

Reusch, T.B., Wood, T.E., 2007. Molecular ecol- ogy of global change. Mol. Ecol. 16:3973-3992.

Saitou, N., Nei, M., 1987. The neighbor-joining method. J. Mol. Evol. 21:1-10.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol Evol. 24:1596-1599.

Tarnchalanukit, T., 1986. Experimental hybridization between cichlids of the families Cichlidae and Pangasiidae in Thailand. Environ. Biol. Fish. 16:317-320.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., 1997. The Clustal X win- dows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.

VASEP, 2014. Vietnam association of seafood exporters and producers. Available from: http://seafood.vasep.com.vn

Velayutham, D., Bellagamba, F., Busseto, M.L., Moretti, V.M., 2010. Pangasianodon hypophthalmus haplotype hp 07-cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial. Accession number GU322354. Available from: http://www.ncbi.nlm.nih.gov/nuccore/282848452

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., Hebert, P.N.D., 2005. Barcoding Australia’s fish species. Phil. Trans. R. Soc. B 360: 1847-1857.

Wolf, C., Rentsch, J., Hubner, P., 1999. PCR- RFLP analysis of mitochondrial DNA: a reliable method for species identification. J. Agr. Food Chem. 47:1350-1355.

Wong, E.H.K., Hanner, R.H., 2008. DNA barcod- ing detects market substitution in North American seafood. Food Res. Intern. 41:828-837.