Multiplex PCR for identification of fish substitution in fish fillet products

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Abstract. Fish substitution in fish fillet products is a concern in food safety and sustainability. There are many reports about the mislabeling problem in fish fillet products. DNA-based identification plays an important role in food safety and traceability. The use of advanced techniques of barcode using multiplex PCR for species identification substitution from fish fillet products are demonstrated in this study. The primer was designed from three different species that are usually labeled in fish fillet products. Each pair of primers were designed to amplify different PCR products that are specifically recognized as different species. We found 11, 6 and 0 samples labeled as Tilapia, Pangasius, and Snapper respectively. This method is suggested for rapid identification because it is efficient and accurate.

Keywords: Multiplex, fish, fillet, substitutions

1. Introduction
Fish provides a significant amount of nutrition that makes fish consumption a choice with people who want to eat healthy food. However, it is reported that fish mislabeling products are common in markets, especially in fish fillet products [1]. There’s a chance that the mislabeled fish could be an endangered species and that problem can damage biodiversity. Accurate identification is important too for food management and consumer welfare. If the species used were not the same as in the label that makes suffer the consumers [2].

Multiplex PCR methods allow using many primers in one reaction. With specific primers in one reaction, the DNA band from the specific sequence will appear. It reduces the cost of reagent and time in analyzing species from one product of fish fillet [3, 4]. In this study, these methods are used to identify the substitution of fish fillet products.

2. Materials and methods

2.1. Sample collecting around Jabodetabek
Samples were collected from traditional and modern markets around Jabodetabek. We choose different species labels from every product. There were unlabeled products found and we collect it. There is Pangasius, Snapper, Tilapia and Gindara in the species label name (table 1).
Table 1. Result of samples collecting around Jabodetabek.

| Location         | Code | Species labelled | Unlabelled |
|------------------|------|------------------|------------|
| East Jakarta     | JT   | JT1, JT4, JT6    | -          |
| West Jakarta     | JB   | -                | JB3        |
| South Jakarta    | JS   | JS5, JS9         | JS6        |
| North Jakarta    | JU   | JU4              | JU2, JU3   |
| Center Jakarta   | JP   | JP4, JP5         | JP3        |
| Bogor            | B    | B2, B3, B4, B5   | B1         |
| Depok            | D    | D1, D3           | D2         |
| Bekasi           | BK   | BK2, BK5         | BK1        |
| Tangerang        | T    | T3, T5           | T4         |

2.2. DNA isolation
The DNA was isolated using chelex resin methods the resin was diluted in pure water and very small pieces of fillet were cut and mixed in the tube with the diluted chelex. The sample was incubated in 60 °C for 20 minutes and then incubated in 103 °C for 25 minutes. The supernatant was taken out and put in the new tube.

2.3. Primer design for 3 species (Pangasius, Tilapia, and Snapper)
Three species were chosen as the model because almost every labeled product was labeled with those species. These three groups were not harmful to be consumed and not from endangered species. So it is assumed that unlabeled products contain fish from those three groups.

All the reference sequences for primer design were retrieved from NCBI based on previous studies. Nine sequences of tilapia (AF328851, AF296493, AF328854, AF328843, AF296467, AF484717, AF485083, AY833459, AY833481) [5], four sequences of pangasius (EU148557, EU148558, EU148559 and EU148560) [6], and ten sequences of snapper (MG021099, MG022139, MG022140, MG022141, MG022142, MG022143, G022144, MG022145, MG022146 and MG022147) [7] were retrieved for designing 3 pairs of primer species of each group species. Each primer was analyzed for GC content, melting temperature, and specificity. Each pair of primer was designed to amplify different PCR products that is easily recognized as different species.

2.4. Multiplex PCR
The multiplex PCR was carried out with three primers that were previously designed. Each 25 μL PCR reaction included 5 μL KAPA Multiplex 2G Master Mix, 12.5 μL nuclease-free water, and six primers 0.75 μL for each primer.

2.5. Electrophoresis gel
Electrophoresis was done to check the results. The appearance of bands were observed in the gel by in silico analysis for the designed primer.

3. Results and discussion
The results of in silico analyses were six primers that were used in multiplex PCR. This result was used as a reference for temperature optimization. For optimization, we used ten temperatures, from 50 °C to 60 °C, according to table 2. Figure 1 below shows the result is 57.1 °C.
Table 2. Results of *in silico* analyses for primer design.

| No | Primer name | Sequences 5’-3’ | Tm (ºC) | GC (%) | Specificity                                      |
|----|-------------|------------------|---------|--------|-------------------------------------------------|
| 1  | TLP-F       | CCGAGCTCTGCTTCATGC | 61      | 63.2   | No secondary structure; No primer dimer         |
|    |             |                   |         |        |                                                 |
| 2  | TLP-R       | CTTGACCTTCAAGAACCCTT | 55.3    | 45     | Moderate secondary structure; No primer dimer   |
|    |             |                   |         |        |                                                 |
| 3  | PGS-F       | GCCCCCTGATATGGCATTCCC | 61.4    | 60     | Moderate secondary structure; No primer dimer   |
|    |             |                   |         |        |                                                 |
| 4  | PGS-R       | GAAAAGGTGTGATATAGGA | 51.1    | 35     | No secondary structure; No primer dimer         |
|    |             |                   |         |        |                                                 |
| 5  | SNP-F       | GCTGGGACTGGATGAACGTT | 59.3    | 55     | Moderate secondary structure; No primer dimer   |
|    |             |                   |         |        |                                                 |
| 6  | SNP-R       | AGGGAAAGAAGGAGCAGGAC | 59.3    | 55     | No secondary structure; No primer dimer         |
|    |             |                   |         |        |                                                 |

The number of samples that PCR was running with a multiplex primer is 30 samples. These samples were collected from different brands from different markets. Most of the samples had species labels. There are 11 of 30 samples collected from unlabeled species fish fillet products, as seen in table 1.

Table 3 shows that from 30 samples, 17 samples had positive results with multiplex PCR. From 17 positive samples, 11 were the Tilapia group, 6 were the Pangasius group, and the Snapper group was not detected. The results which can be seen in table 4 were determined from the in silico analysis on the band size from the electrophoresis gel.
Figure 2 shows how the bands looks like in electrophoresis gel. Almost all of the unlabeled products had negative results. This could be because the samples contain none of the three groups. Snapper was not detected maybe there so it was possible that no Snapper used as fish products or mislabeled. All the positive results in Pangasius and Tilapia were labeled appropriately. There were mislabeled products labeled as Pangasius but gave negative results. There is a product that is unlabelled but gives a positive result in Pangasius. This indicates that there are unlabelled products that use the same species as the labeled products but most of them are not one of these three groups.

This method is more efficient and accurate for rapid identification because no gene sequencing of the sample is necessary. The specific band in the electrophoresis gel is specific to the genus of the fillet. So we can isolate many samples and know the results simultaneously. But the flexibility of these methods is limited to these three genera.

**Table 3.** Results of multiplex PCR.

| No. | Sampling location | Primer (Tilapia group) | Primer (Pangasius group) | Primer (Snapper group) | Not detected |
|-----|-------------------|------------------------|--------------------------|------------------------|-------------|
| 1   | North Jakarta     | JU4                    | -                        | -                      | JU2*, JU3*  |
| 2   | East Jakarta      | -                      | JT6, JT1                 | -                      | JT4        |
| 3   | South Jakarta     | JS9                    | JS5                      | -                      | JS6*       |
| 4   | Central Jakarta   | JP4                    | JP5                      | -                      | JP3*       |
| 5   | West Jakarta      | -                      | -                        | -                      | JB3*       |
| 6   | Tangerang         | T3, T5                 | -                        | -                      | T2*, T4*   |
| 7   | Bogor             | B3, B4, B2             | B5                       | -                      | B1*        |
| 8   | Bekasi            | BK 2                   | BK5                      | -                      | BK1*, BK4  |
| 9   | Depok             | D1, D3                 | -                        | -                      | D2*, D4    |

*Unlabeled species fish fillet products

**Tabel 4.** Primer product size and species target.

| No | Primer | Product size (bp) | Target               |
|----|--------|-------------------|----------------------|
| 1  | TLP    | 339               | Tilapia species group|
| 2  | PGS    | 441               | Pangasius species group|
| 3  | SNP    | 233               | Snapper species group|

Figure 2. Result of Multiplex PCR.
4. Conclusion

Samples that give positive result are safe for consumers. Samples that give negative results indicate that fish species used was not from Tilapia, Pangasius, and Snapper group. That may be harmful to the consumers or the environment because there is a chance that species used in unlabeled products are from endangered species or contain a toxin. They need to be sequenced to know what species was used for the products, especially for all the unlabeled products.

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