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Molecular Evidence for Determination Cryptic Species of Indonesian Swamp Eel Populations using Denaturing Gradient Gel Electrophoresis (DGGE)

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Abstract. Swamp eel is the Indonesian freshwater fish that should be understood and preserved due to their high economic potency in aquaculture. Additionally Indonesian swamp eel is commonly accepted to the species described as Monopterus albus (Synbranchiformes: Synbranchidae). However, the knowledge of genetic variation of this species from Indonesia is quite limited. Hence, in this study, we used the Denaturing Gradient Gel Electrophoresis (DGGE) and surveyed the genetic variation of cytochrome c oxidase subunit I (COI) in Indonesian swamp eels. Total of 300 samples of the swamp eel fish were collected from 15 populations in Indonesia. The DGGE results represented two genetic patterns (A and B) of the COI variations. In addition, the results revealed that five populations showed pattern A and six populations exhibited pattern B while four populations showed both patterns A and B of the COI variations. Therefore, the result supported the molecular data that the Indonesian swamp eel is a cryptic species complex. In addition, the distribution of swamp eel’s COI patterns in Indonesia approves that DGGE is a rapid and sensitive method for the detection of a genetic variation of the fish.

Keyword: cryptic species, DGGE, swamp eel.

INTRODUCTION

Indonesia is an archipelago in Southeast Asia consisting 17 508 islands and is considered to be a megalobiodiversity country next to Brazil. The country straddles the equator and the two of the world’s seven major biogeographic regions, the Oriental and Australasian. The archipelago is also one of the world’s centres of species diversity including fishes [1]. For fishes, the total number of freshwater fish species is about 1 400 or 7 % of total global species [1]. The number of freshwater species could be increased due to systematic sampling and associated taxonomic studies.

Fish of the family Synbranchidae group is economically important freshwater fishes in worldwide including Indonesia. Within this family, the genus Monopterus are especially popular due to their reputation as delicious food, their ability to survive and grow in poorly oxygenated waters and to be transported live. Approximately 10 species of Monopterus are currently recognized, with the majority of species inhabit in Asia especially India and one species found in Africa [2–6]. Of the nine species presently recognized in Asia, one species is native to Indonesia which is Monopterus albus Zuiew [2, 6].

The fish is morphologically unique known to reach about 30 cm to 100 cm in length and easily recognized by the cylindrical snake-like body with tapered tail and small eyes. Its body colour is brown above and white or light-
brown below. In adults, paired fins are lacking, and the dorsal, caudal and anal fins are reduced. The gill openings are merged into a single slit underneath the head while the mouth is large and protractile and both upper and lower jaws have tiny teeth for eating. The skin of the fish species produces a thick mucous layer making the eels difficult to hold [2, 7].

The species occurs throughout Indonesia including Sumatra, Java, Bali, Lesser Sundas, Sulawesi, and the Mollucas, and is thought to occur widely in south-east Asia and extending into India and China [6, 7]. A paradox of this extensive distribution is that the species breeds in freshwater (nondiadromous) and is, therefore, likely to have any limited powers of dispersal. Reproductive isolation among populations leads to genetic divergence and over sufficiently long periods of time can lead to speciation. Indeed, this life history is thought to be an important factor to account for the high diversity of swamp eel’s world wide including Indonesia.

The previous molecular genetic studies of the swamp eel have been conducted using isozymes [8]; RAPD [9], direct sequencing of mitochondrial DNA regions [10–12] and the results showed that the fish has a high degree of genetic variation. In addition, the previous studies by Matsumoto et al. [10] and Collins et al. [11] only included swamp eels from Jakarta and Yogyakarta thus the knowledge of genetic variation of this species from Indonesia is quite limited.

In this study, we surveyed and assessed genetic variation of the Indonesian swamp eels using Denaturing Gradient Gel Electrophoresis (DGGE) of mitochondrial cytochrome c oxidase subunit I (COI) gene. Denaturing Gradient Gel Electrophoresis (DGGE) is a very powerful genetic fingerprinting tool developed by Fischer and Lerman [13]. DGGE utilizes a PCR step to amplify the target DNA fragment, which then applied to a denaturation gradient to separate any sequence variants present within the PCR products based on their melting characteristics [13, 14]. This melting profile is totally dependent on the sequence of the DNA molecule, therefore allowing for the identification of DNA fragments differing by as a single nucleotide [15, 16].

**MATERIALS AND METHODS**

**Sample Collection and Storage**

Total of 300 samples of the swamp eel fish were collected from 15 populations in several regions of Indonesia (Table 1). A muscle tissue was dissected from each partially thawed fish and placed into 1.5 mL screw top cryogenic vials and preserved in 95 % ethanol. Fish tissue samples were then transferred to Charles Darwin University, Australia with Letter of Approval from Centre for Fish Quarantine, Ministry of Marine and Fisheries, Republic of Indonesia. All of the specimens were then stored at -20 °C. The research has been approved by The Charles Darwin University Animal Ethics Committee with Project Reference No. A10028.

**DNA Extraction and Amplification**

Total genomic DNA were sequentially extracted from muscle tissue of each specimen preserved in 95 % ethanol using DNeasy Tissue Kit (QIAGEN), according to the manufacture’s protocols. A short highly variable fragment of the COI was selected for DGGE analysis. This fragment is approximately 280 bp and allowed discrimination of the two divergent forms of swamp eel. The DGGE procedure required one of the primers to be modified at the 5’ end with a GC-Clamp. The two primers designed for DGGE analysis are EELCOIF and EELCOIRGCCLAMP. The KAPA2G Robust PCR Kit (Kapa Biosystems) was used for the polymerase chain reaction (PCR). PCR reactions were performed in 25 μL final volume containing 10 ng to 100 ng of genomic DNA, 0.2 mM of each dNTP, 2 mM MgCl₂, 0.014U Taq Polymerase, 0.6 μM of each primer and 1 × PCR reaction buffer. Reactions were amplified using the following cycling conditions: an initial denaturation 94 °C for 1 min; 2 cycles of 30 s at 94 °C, 30 s at 48 °C, 30 s at 72 °C; 2 cycles of 30 s at 94 °C, 30 s at 49 °C, 30 s at 72 °C; 33 cycles of 30 s at 94 °C, 30 s at 50 °C, 30 s at 72 °C and a final extension of 10 min at 72 °C.
**TABLE 1.** Locations of sampled swamp eels (*M. albus*) in Indonesia

<table>
<thead>
<tr>
<th>Location &amp; Code</th>
<th>Grid Reference</th>
<th>Sample Size (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Padang (PDG), WS</td>
<td>S 0° 42' 11.27&quot; ; E 100° 27' 0.81&quot;</td>
<td>20</td>
</tr>
<tr>
<td>Payakumbuh (PYK), WS</td>
<td>S 0° 18’ 37.97” ; E 100° 35’ 32.17”</td>
<td>20</td>
</tr>
<tr>
<td>Depok (DPK), WJ</td>
<td>S 06° 23’ 30.63”; E 106° 48’ 21.74”</td>
<td>20</td>
</tr>
<tr>
<td>Ciomas (CMS), WJ</td>
<td>S 06° 33’ 54.94”; E 106° 44’ 31.97”</td>
<td>20</td>
</tr>
<tr>
<td>Petungkriyono (PKY), CJ</td>
<td>S 07° 08’ 48.52”; E 109° 43’ 24.96”</td>
<td>20</td>
</tr>
<tr>
<td>Kemiri (KMR), CJ</td>
<td>S 07° 39’ 19.08”; E 109° 53’ 11.19”</td>
<td>20</td>
</tr>
<tr>
<td>Planggu, Trucuk (PLG), CJ</td>
<td>S 07° 43’ 31.12”; E 110° 39’ 25.69”</td>
<td>20</td>
</tr>
<tr>
<td>Tawangmangu (TWG), CJ</td>
<td>S 07° 39’ 31.57”; E 111° 08’ 2.01”</td>
<td>20</td>
</tr>
<tr>
<td>Brosot (BRS), Yogyakarta</td>
<td>S 07° 56’ 42.85”; E 110° 13’ 48.33”</td>
<td>20</td>
</tr>
<tr>
<td>Gamping (GMP), Yogyakarta</td>
<td>S 07° 46’ 34.48”; E 109° 19’ 45.47”</td>
<td>20</td>
</tr>
<tr>
<td>Palbapang (PLB), Yogyakarta</td>
<td>S 07° 54’ 21.21”; E 110° 19’ 15.97”</td>
<td>20</td>
</tr>
<tr>
<td>Walikukun (WLK), EJ</td>
<td>S 07° 29’ 58.48”; E 111° 13’ 43.06”</td>
<td>20</td>
</tr>
<tr>
<td>Negara (NGR), Bali</td>
<td>S 08° 18’ 53.96”; E 114° 36’ 08.97”</td>
<td>20</td>
</tr>
<tr>
<td>Narmada (NRM), WNT</td>
<td>S 08° 35’ 27.70”; E 116° 12’ 01.32”</td>
<td>20</td>
</tr>
<tr>
<td>Sindendreng (RPG), SS</td>
<td>S 03° 50’ 47.36”; E 119° 49’ 24.96”</td>
<td>20</td>
</tr>
</tbody>
</table>

**DGGE Optimization and Procedures**

Optimal DGGE profile resolution is influenced by several factors including primer design, electrophoretic conditions, gel composition, and staining methods. These several factors were tested to determine the optimal DGGE conditions for assessing genetic variation of Indonesian swamp eels. Gels with different acrylamide: bis-acrylamide ratios (29:1 or 37.5:1) were tested to determine which of the two ratios generated the highest resolution banding patterns. DGGE was performed with the Ingeny PhorU-2 apparatus (Ingeny International, The Netherland) as per manual instruction using a gradient mixer (Ingeny International, The Netherland) to form the linear denaturation gradient. The gel was polymerized by adding 120 μL of 20 % (w/v) ammonium persulphate and 12 μL of N,N,N,N-tetramethylethylenediamine to each 24 mL of polyacrylamide solution. Approximately 50 ng of each PCR amplicon mixed with an equal volume of loading buffer (0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol and 15 % (v/v) glycerol was loaded on 6.5 % polyacrylamide gels in TAE (1 ×) buffer. Optimal separation was achieved with a parallel denaturing gradient of urea-formamide ranging from 20 % to 40 % (100 % corresponded to 7 M urea and 40 % v/v formamide). Gels were run 17 h at 60 °C and 75 V and stained with SYBR Gold (8 μL in 50 mL of 1 × TAE) for 15 min. The banding pattern was then photographed on a UV transilluminator and determined using Quantity One 1-D analysis software.

**RESULTS AND DISCUSSION**

The result showed that the effect of the 37.5:1 ratio of acrylamide : bis-acrylamide was better than that of 29:1 ratio on banding profile resolution. Therefore, this study routinely used 37.5:1 acrylamide : bis-acrylamide gels for the DGGE assays. The DGGE results represented two genetic patterns (A and B) of the COI variations (Fig. 1). Five populations revealed pattern A (PKY, PLG, TWG, WLK, NRM) and six populations exhibited pattern B (PDG, PYK, DPK, CMS, KMR, RPG) while 4 populations showed both patterns A and B (BRS, GMP, PLB, NGR) of the COI variations (Fig. 2 and Table 2).
FIGURE 1. DGGE analysis of Indonesian swamp eels (*M. albus*) using the primer sets for the COI mitochondrial gene. The numbers correspond to the patterns obtained in this study (number 1–10 correspond to pattern B and number 11–15 correspond to pattern A). Sample number 1–2: PDG, 3–4: PYK, 5–6: DPK, 7: CMS, 8–9: KMR, 10: RPG, 11: PKY, 12: PLG, 13: TWG, 14: WLK, 15: NRM.

These preliminary findings have progressed sufficiently to present results for genetic variation in mitochondrial COI gene of Indonesian swamp eels. Therefore, the DGGE method has proven highly efficient for the reliable diagnosis of the genetic variability of the fish. The results have important implications for evaluating the possibility of cryptic species of Indonesian swamp eels because the life history of the fish spending their entire lifecycle in freshwater habitat and have limited powers of dispersal.

The results also revealed that the three populations of swamp eels from Yogyakarta (BRS, GMP, and PLB) have both pattern A and B in each population. These findings supported [10] investigation reporting that swamp eels from Yogyakarta have a high genetic diversity. In addition, the results showed that there is evidence for translocations as both patterns of the Indonesian swamp eel are present. Furthermore, the result showed evidence of population-level genetic variation, but this needs a further study using microsatellite loci to assess the extent and significance of this variation.
FIGURE 2. Distributions of COI-DGGE patterns of swamp eels (*M. albus*) in Indonesia (the abbreviation see Table 1)

<table>
<thead>
<tr>
<th>Population</th>
<th>Number of Sample with Pattern A</th>
<th>Number of Sample with Pattern B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Padang (PDG)</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Payakumbuh (PYK)</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Depok (DPK)</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Ciomas (CMS)</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Petungkriyono (PKY)</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Kemiri (KMR)</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Planggu, Trucuk (PLG)</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Tawangmangu (TWG)</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Brosot (BRS)</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>Gamping (GMP)</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Palbapang (PLB)</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>Walikukun (WLK)</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Negara (NGR)</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Narmada (NRM)</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Sindendreng (RPG)</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>

TABLE 2. Identification of genetic patterns of Indonesian swamp eels (*M. albus*) based on DGGE of COI mitochondrial gene variation
CONCLUSION

This study surveyed and assessed genetic variation of the Indonesian swamp eel using Denaturing Gradient Gel Electrophoresis (DGGE) of the mitochondrial cytochrome c oxidase subunit I (COI) gene. To our knowledge, this is the first study evaluating the DGGE apparatus to examine genetic variation of the swamp eel. The result showed that the distribution of swamp eel’s COI patterns in Indonesia approve that DGGE is a rapid and sensitive method for the detection of a genetic variation of the fish. Our results recommend the use of the same system throughout an experiment if multiple gel-to-gel analysis is required.

ACKNOWLEDGMENT

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