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Genetic structure of locally threatened cyprinid, *Osteochilus melanopleurus*, in Peninsular Malaysia River systems inferred from mitochondrial DNA control region

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ABSTRACT

The genetic structure of the locally threatened cyprinid *Osteochilus melanopleurus*, collected from four locations (Gerik, Manong, Bernam and Kahang) in Peninsular Malaysia River systems, was assessed based on partial sequences of the control region (D-loop) mitochondrial DNA. A partial D-loop of 364 base pair gene was extracted from 88 individuals through PCR amplification and direct sequencing. The results show that haplotype diversity ($hd = 0.7956–0.9485$) was high, with low nucleotide ($\pi = 0.0045–0.0080$) variation for each of the populations examined. A molecular variance analysis (AMOVA) revealed few differences between the four populations. A Tajima D test and Fu *Fs* test were used to investigate the neutrality of each population, revealing that the all examined populations might experience recent population expansion or the bottleneck effect. A phylogeny tree and mismatch distribution analysis provided further evidence that three of four populations had recently expanded, as well as indicating that the Kahang population had in contrast remained stable. Overall, this study concludes that the populations of *O. melanopleurus* distributed throughout the river systems of Peninsular Malaysia probably originated from a single ancestral source during the last glaciation period, and should therefore be treated as a single evolutionary unit in any conservation efforts.

1. Introduction

Freshwater fish are one of the groups of vertebrates that are in serious decline throughout the world. Anthropogenic activities such as pollution, overharvesting, dam construction and habitat loss (Chong et al., 2010; Taylor et al., 2000) have led to a steady increase in the number of threatened freshwater fish species. There are vast ranges of river systems in Malaysia, flowing into both the Malacca Straits and the South China Sea, which contain a large diversity of fish fauna. Chong et al. (2010) reported that a significant proportion of primary freshwater fish in Malaysia up to 30% of 470 identified species were facing a

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high risk of extinction. The number of threatened freshwater fish species is still considered moderate at present, but the current growth in dam construction, uncontrolled pollution and other pressures are likely to increase the extinction threats to freshwater fishes, unless serious conservation efforts are made.

This study seeks to assess the genetic structure of one primary freshwater cyprinid with limited distribution. *Osteochilus melanopleurus*, locally called kelabau or kelabu, is classified as a moderately threatened species in Peninsular Malaysia as a result of overharvesting and population disruption due to pollution. Classified under the family Cyprinidae, the largest family found in Malaysia, *O. melanopleurus* is a benthic–pelagic species which inhabits the water column just above the bottom, feeding on phytoplankton, filamentous algae and benthic algae (Rainboth, 1996). High demand for this species has led to a steady increase in price: from RM4.00 per kilogram in 1983 (Aizam et al., 1983) to RM12.00 to RM 28.00 per kilogram more recently in certain parts of Peninsular Malaysia, based on personal communication with local fishermen.

Understanding the genetic background is of crucial importance for the conservation and management of wild species. Molecular markers have been widely used in genetic studies of populations for this purpose (Sun et al., 2012; Zhao et al., 2013). Mitochondrial DNA (mtDNA) is an extra-nuclear DNA that has proven to be a useful molecular marker in fish population studies and evolutionary genetics, because of its small molecular weight, maternal inheritance, relatively rapid base substitution rate, and lack of recombination (Avise et al., 1987). The control region (D-loop) is a non-coding region and is recognized as the most variable portion of the mtDNA genome. It is commonly variable at the intraspecific level, making it widely used in studies of genetic variability among populations and phylogenetic analysis (Donaldson and Wilson, 1999; Liang et al., 2011; Zhao et al., 2013).

To date, there has been only one single publication presented on the reproductive process in nature of *O. melanopleurus* (Aizam et al., 1983). This scarcity of information on the species is an obstacle to future measures to manage and conserve it. The aim of this study is to address this lack of information on the targeted species with particular reference to its genetic variation and population dynamics.

2. Materials and methods

2.1. Sampling

A total of 88 individuals of *O. melanopleurus* were successfully collected from four study sites, namely Gerik, Manong, Bernam River and Kahang River (Fig. 1). As the cyprinid is classified as locally threatened, we purchased all the individuals from local fishermen at priced ranging from RM12 to RM40 per kilogram. After collection, tissues were extracted from each individual and were kept in a freezer at −20 °C pending further processing.

2.2. DNA extraction and sequence analysis

Genomic DNA was extracted using a standardized extraction kit, following the protocols set by the manufacturer (MACHEREY NAGEL). DNA was visually inspected for quality and quantity on 1.2% agarose gel. Two universal vertebrata conserved D-loop primers; L19 (5′-CCACTAGCTCCCAAAGCTA-3′) (Bernatchez et al., 1992) and 98H (5′-CCTGAAGTAGGAACCAGA-3′) (Meyer et al., 1990) were used to amplify targeted gene. PCR was performed in 10 μl reactions, consisting of a Promega reaction buffer or ABI 10× buffer II (Applied Biosystems Inc.); 1.0–3.0 mM $MgCl_2$; 0.8 mM dNTPs; 0.1 l M each primers, 0.8 units Taq DNA polymerase; and 20–60 ng genomic DNA and performed on a Mastercycler EP PCR machine (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C denaturation for 30 s, 30 s at the required annealing temperature, and 72 °C extension for 30 s. The annealing temperature for mtDNA PCR was 55.4 °C. The PCR products were then subjected to a 1% agrose gel before the single targeted band was excised for purification. The gel band containing the specific D-loop gene was cleaned up using a gel purification kit in accordance with the manufacturer's (MACHEREY NAGEL) protocol. The purified DNA was sequenced directly using an ABI platform 5330 XL automatic sequencer.

Consensus sequences for all individuals were aligned using CLUSTAL X software (Thompson et al., 1997), and were visually edited. Arlequin 3.0 (Excoffier et al., 2005) and DnaSP 5.0 software (Librado and Rozas, 2009) were used to calculate the genetic characteristics, including the nucleotide composition, number of polymorphic sites (S), haplotype diversity (Hd) (Nei, 1987), and nucleotide diversity (π) (Lynch and Creasef, 1990).

The phylogeny tree was constructed based on the neighbour-joining (NJ) method (Saitou and Nei, 1987) using the Tamura-Nei model which modulated based on the Model Test (Posada and Crandall, 1998). A median-joining method was constructed to predict the phylogenetic and geographical relationships of the retrieved haplotypic sequences using Network 4.1 (Rohl, 2003) based on the reduced median approach. *Osteochilus salsburyi* complete mtDNA sequence was used as outgroup (D-loop region only) from Genbank (Accession number: JX220892).

The genetic differentiation between populations was assessed based on an analysis of molecular variance (AMOVA), and was calculated using Arlequin 3.0 (Excoffier et al., 2005). Fixation indices (FST) were calculated on the basis of the information from the haplotypes and their frequencies. The significance of the fixation index was tested by 1000 permutations of the data set. Finally, a Tajima D test (Tajima, 1989), Fu Fs test (Fu, 1997) and mismatch distribution analysis were carried out to evaluate the demographic history pattern of the populations examined, using Arlequin 3.0 and DnaSP 5.0 software.