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The mitochondrial genome of Indonesian coelacanth *Latimeria menadoensis* (Sarcopterygii: Coelacanthiformes) and divergence time estimation between the two coelacanths

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Abstract

We determined the whole mitochondrial genome sequence for Indonesian coelacanth *Latimeria menadoensis*. The genome content and organization were identical to that of typical vertebrates including Comoran coelacanth, *Latimeria chalumnae*. The overall nucleotide differences between the two species (excluding the control region) was 4.28%. The divergence time between the two species was estimated using whole mitochondrial genome data from the two coelacanths and 26 actinopterygians that represent major actinopterygian lineages plus an outgroup. Partitioned Bayesian analyses were conducted with the two data sets that comprised concatenated amino acid sequences from 12 protein-coding genes (excluding ND6 gene) and concatenated nucleotide sequences from 12 protein-coding genes (without 3rd codon positions), 22 transfer RNA genes, and two ribosomal RNA genes. The molecular clock analysis was also conducted with the concatenated amino acid sequences from the 12 protein-coding genes after removing faster or more slowly evolving sequences. Using the sarcopterygian–actinopterygian split as a calibration point (450 Mya), divergence time estimation between *L. menadoensis* and *L. chalumnae* fell in the range of 40–30 Mya, which is much older than those of the previous studies (<6.3 Mya). Assuming that the most recent ancestor of *Latimeria* was distributed continuously along the deep coasts of Africa through Eurasia, our estimate is in agreement with the hypothesis that the collision of India with Eurasia (50 Mya) and the subsequent siltation caused by the formation of major rivers resulted in a coelacanth habitat disjunction that allowed populations on either side of India to diverge.

Keywords: Indonesian coelacanth; Partitioned Bayesian analysis; Molecular clock; Basal Actinopterygii; Mitogenome

1. Introduction

Coelacanths were believed to have gone extinct more than 80 Mya until the sensational rediscovery of one surviving member of this lineage, *Latimeria chalumnae*, in 1938 (Thomson, 1991). Since then, more than 200 coelacanths have been caught off the Comoro archipelago near the eastern coast of Africa in the Indian Ocean (Forey, 1998). During the period of September 1997 through July 1998, two coelacanths were captured off the coast of Manado, Sulawesi, Indonesia, some 10,000 km east of the southwestern Indian Ocean (Erdmann et al., 1998). These coelacanths are the first individuals recorded from a location outside the western Indian Ocean. The extensive interviews with Indonesian fishermen, combined with the vast distance from the Comoro archipelago, supported the idea that the Indonesian

Abbreviations: ATPase 6 and 8, ATPase subunits 6 and 8; bp, base pair(s); COI–III, cytochrome *c* oxidase subunits I–III; cyt *b*, cytochrome *b*; DMSO, dimethyl sulfoxide; Mya, million years ago; Myr, million years; ND1–6, 4L, NADH dehydrogenase subunits 1–6, 4L; PCR, polymerase chain reaction; tRNA, transfer RNA; 12S rRNA and 16S rRNA, 12S and 16S ribosomal RNA.

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coelacanths are part of an established north Sulawesi population, and not simply waifs from the Comoran population (Forey, 1998).

Taxonomic confusion raised by overlapping morphological variations has posed problems in relation to coelacanth dispersal and biogeography. Pouyaud et al. (1999) described the Indonesian coelacanth as a new species, *Latimeria menadoensis*, based on nine morphological and meristic differences. However, Erdmann et al. (1999) suggested that the Indonesian coelacanth is morphologically similar to the Comoran coelacanth based on a preliminary comparison of external morphological measurements. Based on a survey of the literature, Holder et al. (1999) also stated that the case for morphological differentiation of *L. menadoensis* is much more tenuous than originally reported (Pouyaud et al., 1999) and concluded that one important morphological character that should be considered in future examination is that of scale ornamentation.

Two independent research groups have published results from divergence time estimation of the two coelacanths using partial mitochondrial gene sequences (6.3–4.7 Mya: Holder et al., 1999; 1.3 Mya: Pouyaud et al., 1999). Nevertheless, divergence time between the two coelacanths has remained ambiguous. In general, lineage-specific variation in rate of molecular evolution complicates molecular dating because a calibration rate estimated from one lineage may not be an accurate representation of the rate in other lineages (Bromham and Penny, 2003). These two studies, however, used rates of evolution from amphibians or teleosts for estimation without conducting the test of rate variation among lineages. Moreover, estimation of divergence time is generally more difficult than reconstruction of a phylogenetic tree, because no gene would evolve at a constant rate (Glazko and Nei, 2003). Considering the effect of rate variation among small number of genes, it is no wonder that the analyses based on partial mitochondrial gene sequences may be significantly biased.

In this study, we determined whole mitochondrial genome for Indonesian coelacanth *L. menadoensis* and compared the new sequence to that already reported for Comoran coelacanth *L. chalumnae*. Recent authors have used many genes to estimate divergence times in the hope of reducing the effect of rate variation (Nei and Glazko, 2002). We used whole mitochondrial genome sequences to estimate the divergence time between the two coelacanths because they have been demonstrated in recent studies as being useful for estimating the divergence times among basal lineages within tetrapods (Kumazawa et al., 2004) and within primates (Schrago and Russo, 2003). Two distinct methodologies were employed to estimate divergence time: the partitioned Bayesian approach and the molecular clock approach. Based on the molecular evidence, we evaluated several alternative hypotheses about the speciation of the two coelacanths.

2. Materials and methods

2.1. DNA extraction

Genomic DNA of the *L. menadoensis* was extracted from pieces of gills that were preserved in DMSO or ethanol by using the standard protocol.

2.2. PCR and sequencing

The mitochondrial genome of the *L. menadoensis* was amplified in the entirety using a long PCR technique. Four fish-versatile long PCR primers (S-LA-16S-L+H1065-12S and L12321-Leu+S-LA-16S-H; for locations of these primers, see Miya and Nishida, 2000) were used to amplify the entire mitochondrial genome in two reactions.

The long-PCR products were diluted with TE buffer (1:19) for subsequent use as PCR templates, except for a region intervening between the two long-PCR primers (between S-LA-16S-H and S-LA-16S-L), in which total genomic DNA was used alternatively. A total of 81 fish-versatile and five species-specific PCR primers (Supplement) was used in various combinations to amplify contiguous, overlapping segments of the entire mitochondrial genome for *L. menadoensis*. Five species-specific primers were designed in cases where no appropriate fish-versatile primers were available. Long PCR and subsequent short PCR were carried out as previously described (e.g. Miya and Nishida, 2000; Inoue et al., 2003).

Double-stranded PCR products, purified using a Pre-Sequencing Kit (USB), were subsequently used for direct cycle sequencing with dye-labeled terminators (Applied Biosystems). Primers used were the same as those for PCR. All sequencing reactions were performed according to the manufacture's instructions. Labeled fragments were analyzed on a Model 3100 DNA sequencer (Applied Biosystems).

2.3. Sequence analysis

The DNA sequences were edited and analyzed with EditView ver. 1.0.1, AutoAssembler ver. 2.1 (Applied Biosystems), and DNASIS ver. 3.2 (Hitachi Software Engineering). Locations of the 13 protein-coding genes were determined by comparisons of DNA or amino acid sequences of bony fish mitochondrial genomes. The 22 tRNA genes were identified by their cloverleaf secondary structures and anticodon sequences. The two rRNA genes were identified by sequence similarity. Sequence data is available from DDBJ/EMBL/GenBank with accession number AP006858.

2.4. Alignment

The mitochondrial genome sequences from the Indonesian (this study) and Comoran (Zardoya and Meyer, 1997)