ABSTRACT: The objective of this study was to reconstruct phylogenetic relationships of gibbons (3 genera, 11 species) deduced from complete sequenced mitochondrial genome sequences. According to conserved (C) / variable (V) sites ratio test, 9 selected protein coding genes were combined into a sequence with 9356 bases long including gaps. A resolved phylogenetic tree was obtained for the mitochondrial genome in the maximum-likelihood and maximum-parsimony analyses. In accordance with all previous morphological and molecular evidence, our results clearly supported monophyly of family Hylobatidae with predominantly strongly supported and each of the three genera with high support based on these coding genes. Among three genera, first Nomascus, next Symphalangus and at last Hylobates diverged, which identical to other previous research based on the whole mitochondrial genome. Our phylogenetic relationships of Nomascus group accord with Chan et al. (2010), only support values of a node of N. gabriellae (90%) were slight lower than Chan's result (97%). Among genus Hylobates, in our result, 6 species are involved and H. pileatus, H. lar, H. klossii, H. agilis, H. moloch and H. muelleri diverged sequentially.

KEY WORDS: Hylobatidae; Gibbons; Phylogeny; Mitochondrial genomes
1. GENERAL INSTRUCTIONS

Gibbons are small arboreal apes in the family Hylobatidae, which occur in tropical and subtropical rainforests of the mainland and islands from eastern Bangladesh and northeast India to southern China and Indonesia (including the islands of Sumatra, Borneo, and Java) (Brandon-Jones et al., 2004). The family historically contained one genus, but now is split into four well-recognized genera and 17 species. Primarily on the basis of their karyotypes, four major genera have been identified (Hoolock: $2n = 38$, *Hylobates*: $2n = 44$, *Symphalangus*: $2n = 50$, and *Nomascus*: $2n = 52$) (Brandon-Jones et al., 2004; Carbone et al., 2014), and the monophyly of each group has been inferred from the study on the ND3-ND4 region of mitochondrial DNA (mtDNA) (Takacs et al., 2005).

A number of molecular studies have been performed, including karyotypes (Müller et al., 2003), mitochondrial DNA (mtDNA) (Hayashi et al., 1995; Takacs et al., 2005; Monda et al., 2007; Whittaker et al., 2007; Chan et al., 2010; Matsudaira and Ishida, 2010), Y chromosomes (Chan et al., 2012), *Arthrobacter luteus* (ALU) repeats (Meyer et al., 2012), and short stretches of autosomal sequence (Kim et al., 2011; Wall et al., 2013), the phylogenetic relationships among the four gibbon genera are still confidently unresolved; phylogenetic relationships proposed in the studies using different data were inconsistent (reviewed in (Kim et al., 2011; Wall et al., 2013)).

Recent phylogenetic studies reveal that mitochondrial genomes can provide sufficient resolution for reconstructing a robust phylogeny (Gómez-Carballa et al., 2015; Muisuk et al., 2015; Wang et al., 2015). The use of gibbons’ whole mitochondrial genome sequences may help to resolve their undetermined phylogenetic relationships. The present study aims to resolve the phylogeny of the genus *Hylobates* based on their mt genomes have been sequenced, which provided much stronger support for the resulting phylogenetic reconstruction.

2. MATERIALS AND METHODS

So far, 11 gibbon complete mitochondrial genome sequences were published in Genbank involving 3 genera: *Hylobates* (H. agilis, H. klossii, H. lar, H. moloch, H. muelleri, and H. pileatus); *Nomascus* (N. concolor, N. gabriellae, N. leucogenys, and N. siki) and *Symphalangus* (S. syndactylus) (Table.1). In this study, the 11 published gibbon complete mitochondrial genome sequences were all analyzed.

According to Junqueira et al. (2004), conserved (C) / variable (V) sites ratio was used to estimate the reliability of alignment of 13 protein coding genes, conserved (C) / variable (V) sites were calculated by using MEGA 4.0. Nine genes (COI, COII, COIII, ND1, ND3, ND4, ND5, ATP6, CytB) show $>1$ of C/V ratio were concatenated and used for further phylogenetic analyses (Fig. 1). Four genes (ND2, ND4L, ND6 and ATP8) with a C/V ratio $<1$ were excluded (Fig. 1).

Table 1. List of gibbon complete Mitochondrial genome sequences retrieved from Genbank in this study.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>GenBank ID</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hylobates</em></td>
<td><em>Hylobates agilis</em></td>
<td>NC_014042</td>
</tr>
<tr>
<td></td>
<td><em>Hylobates klossii</em></td>
<td>HQ622788</td>
</tr>
<tr>
<td></td>
<td><em>Hylobates lar</em></td>
<td>NC_002082</td>
</tr>
<tr>
<td></td>
<td><em>Hylobates moloch</em></td>
<td>HQ622783</td>
</tr>
<tr>
<td></td>
<td><em>Hylobates muelleri</em></td>
<td>HQ622780</td>
</tr>
<tr>
<td></td>
<td><em>Hylobates pileatus</em></td>
<td>NC_014045</td>
</tr>
<tr>
<td><em>Nomascus</em></td>
<td><em>Nomascus concolor</em></td>
<td>HQ622808</td>
</tr>
<tr>
<td></td>
<td><em>Nomascus gabriellae</em></td>
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</tr>
<tr>
<td></td>
<td><em>Nomascus leucogenys</em></td>
<td>NC_021957</td>
</tr>
<tr>
<td></td>
<td><em>Nomascus siki</em></td>
<td>AB504751</td>
</tr>
<tr>
<td><em>Symphalangus</em></td>
<td><em>Symphalangus syndactylus</em></td>
<td>NC_014047</td>
</tr>
</tbody>
</table>

To perform phylogenetic analyses with a large data set, 9 selected protein coding genes were combined manually into a sequence with 9356 bases long including gaps and then aligned by Clustal V implemented in MEGA 4.0 (Tamura et al., 2007), which was used for the following analyses. The corresponding sequences of *Pongo pygmaeus* (No: D38115), *Pongo abelii* (X97707), *Gorilla gorilla* (D38114), *Homo sapiens* (AF347015), *Pan paniscus* (D38116), *Pan troglodytes* (D38113) and *Macaca mulatta* (AY612638) were as outgroups.

Mitochondrial phylogeny was inferred from the aligned concatenated data by Maximum Likelihood (ML) and Maximum Parsimony (MP) analysis independently. We used PAUP*4.0b8a (Swofford, 2001) perform ML and MP analyses.

To determine which model of evolutionary change best fit the data for the maximum likelihood analysis, the program JModeltest 0.1.1 (Posada,
was employed, using the hierarchical likelihood ratio test (hLRT) to choose which of 56 models best fit the data. A heuristic maximum likelihood search using 100 random-addition sequences and performing TBR branch swapping was performed with 100 bootstrap replications.

Using the hLRT, the best fitting nucleotide substitution model was HKY + G. This model assumes that transitions are more likely than transversions, that purine and pyrimidine transitions are equally likely, and that the substitution rate follows a gamma distribution.

In the MP analysis, we used unweighted maximum parsimony analysis to find the most parsimonious tree. Nucleotides were treated as unordered and equally weighted characters and the gaps were considered as missing data. A heuristic search algorithm using 100 random-addition sequences and performing TBR branch swapping was performed with 1000 bootstrap replications.

3. RESULTS AND DISCUSSION

Based on 9 concatenated protein coding genes, using Maximum Likelihood (ML) and Maximum Parsimony (MP) methods, two equally likely trees were produced. The family Hylobatidae is monophyletic with predominantly strongly supported (100% bootstrap support) and each of the three genera with multiple species also show monophyly with high support (98-100% bootstrap support). At this point, our result is consistent with all previous result, including numerous morphological and molecular studies on the phylogenetic relationships among Hylobatidae members (Hayashi et al., 1995; Müller et al., 2003; Brandon-Jones et al., 2004; Takacs et al., 2005; Monda et al., 2007; Whittaker et al., 2007; Matsudaira and Ishida, 2010; Kim et al., 2011; Chan et al., 2012; Meyer et al., 2012; Wall et al., 2013).

Among three genera, first Nomascus, next Symphalangus and at last Hylobates diverged. The mitochondrial phylogeny of the three gibbon genera, Nomascus, Symphalangus, and Hylobates, based on the 9 concatenated protein coding genes was identical to that based on the whole mitochondrial genome (Hayashi et al., 1995; Müller et al., 2003; Brandon-Jones et al., 2004; Takacs et al., 2005; Monda et al., 2007; Whittaker et al., 2007; Chan et al., 2010; Matsudaira and Ishida, 2010; Kim et al., 2011; Chan et al., 2012; Meyer et al., 2012; Finstermeier et al., 2013; Wall et al., 2013).

However, previous phylogenetic studies using the whole mitochondrial genome, Matsudaira and Ishida (2010) and Finstermeier et al. (2013) both included only one Nomascus species. The former is Nomascus sp. and the latter is Nomascus leucogenys. In our present study, 4 species of Nomascus were involved, N. concolor, N. gabriellae, N. leucogenys, and N. siki. N. leucogenys and N. siki are shown as sister taxa, with N. gabriellae as the next most closely related. N. concolor is the first diverged species within Nomascus group. Our phylogenetic relationships of Nomascus group accord with Chan et al. (2010), only support values of a node of N. gabriellae (90%) were slight lower than Chan's result (97%).

Among genus Hylobates, in our result, 6 species are involved and H. pileatus, H. lar, H. klossii, H. agilis, H. moloch and H. muelleri diverged sequentially. However, In Chan's result (2010), based on whole mtgenomes removing the control regions, H. muelleri and H. agilis; H. klossii and H. moloch; and H. pileatus and H. lar are shown as sister taxa with high bootstrap values (>97%). Cause we just only involved 9 selected protein coding genes, with some very low bootstrap (38% and 40%), our result might have somewhat unreliable. More newly sequenced the whole mitochondrial genome of gibbons specie may help reconstruct gibbon phylogenetic relationships.
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