



DEVELOPMENT OF SPECIFIC PRIMERS FOR INTER SPECIES PHYLOGENY RELATIONSHIP ON *Crocodylian* sp

Herdhanu Jayanto¹ and Budi Setiadi Daryono²

¹ Kelompok Studi Herpetologi, Faculty of Biology, Universitas Gadjah Mada.

² Genetics Laboratory, Faculty of Biology, Universitas Gadjah Mada,
author h.jayanto@gmail.com

ABSTRACT

Poaching, trafficking, and illegal product trading are classic activities which frequently faced by Crocodylian group. To overcome, laws need supporting methods for a decision of these all activities which threaten crocodile species. This will require species identification that associated to taxonomy classification. Crocodylian species are very similar in morphology. This may result to a false identification especially when working on incomplete specimen. Currently, twenty-four existing Crocodylian species are continuously revised to improve the precise placement and/or acceptance of certain species on Crocodylian classification. Herein we address this issue using *Cytochrome-b*. The idea was to obtain genus specific primer from *Cytochrome-b* and then tested the precision of the designed primers using bioinformatics tools' Primer-BLAST and CLC sequence Viewer 6. The designed primers showed a highly specificity on species level. The phylogenetic tree constructed by is relatively precise compared to reported phylogenetic trees. These specific primers together with the genus specific primers may give valuable and important support for the effective and efficient identification of Crocodylian group.

Keywords: Crocodylian, illegal trading, *Cytochrome-b*, specific primer, bioinformatic

INTRODUCTION

Molecular techniques are considered to be useful tools to answer many biological questions. In which over fifty years they developed, thus majorly become root of bioinformatics in nowadays. Bioinformatics cannot be ignored, it consists million of genetic sequences information database and various valuable analyzing tools (Mount, 2004). We observed mtDNA are popular and widely used in Crocodylian phylogenetic studies, together with their sequences information deposited in GenBank (see McAliley *et al.*, 2006; Li *et al.*, 2007; Meganathan *et al.*, 2010; Meganathan *et al.*, 2011; Meredith *et al.*, 2011).

Poaching, trafficking, illegal product trading are activities which frequently faced by Crocodylian group. To response these problems, laws need a supporting scientific method to penalty these all activities which threaten crocodile species. This required species identification that associated to taxonomy classification. Currently, twenty-four of existing Crocodylian species were continuously revised for the correct placement of species on Crocodylian classification. For instance *Tomistoma schlegelii* from Crocodylidae had experienced two times revision (Willis *et al.*, 2007; Vitt & Caldwell, 2009; Uetz & Hallermann, 2012). As we know Crocodylian species are very similar in morphology. This may lead to identification problems especially when we work on incomplete specimen. Herein we address this issue using *Cytochrome-b* which has been used for animal identification or forensic diagnosis (Hsies *et al.*, 2001; Prusak *et al.*, 2005; Meganathan *et al.*, 2009). The idea was to obtain a genus specific primer from *Cytochrome-b* and then tested the precision of the designed primers using bioinformatics tools' Primer-BLAST and CLC sequence Viewer 6.

MATERIALS AND METHODS

The major work of this study were done *in silico*. The *Cytochrome-b* sequences of available species were retrieved from GenBank NCBI (<http://www.ncbi.nlm.nih.gov/>). The phylogenetic tree constructed from alignment of sequences (Table 1.) with CLC sequence Viewer 6 software. Primer-BLAST (Basic Local Alignment Search Tool) software was used to design primers, followed by arrangement to discover the genus specific primers.

Table 1. Sequences from NCBI which used in this study. The Sequences are from Oaks (2011), used with permission.

Species	Accession number
Crocodylidae	
<i>Crocodylus porosus</i>	JF315290
<i>Crocodylus siamensis</i>	JF315292
<i>Crocodylus novaeguineae</i>	JF315289
<i>Crocodylus johnstoni</i>	JF315260
<i>Crocodylus mindorensis</i>	JF315252
<i>Crocodylus palustris</i>	JF315254
<i>Crocodylus niloticus</i>	JF315270
<i>Crocodylus intermedius</i>	JF315246
<i>Crocodylus moreletii</i>	JF315256
<i>Crocodylus acutus</i>	JF315263
<i>Crocodylus rhombifer</i>	JF315255
<i>Crocodylus suchus</i>	-
<i>Mecistops cathrapactus</i>	JF315280
<i>Osteolaemus tetraspis</i>	JF315272
<i>Tomistoma schlegelii</i>	JF315305
Gavialidae	
<i>Gavialis gangeticus</i>	JF315302
Alligatoridae	
<i>Alligator mississippiensis</i>	JF315322
<i>Alligator sinensis</i>	JF315321
<i>Caiman crocodilus</i>	JF315307
<i>Caiman yacare</i>	JF315314
<i>Caiman latirostris</i>	JF315308
<i>Paleosuchus palpebrosus</i>	JF315319
<i>Paleosuchus trigonatus</i>	JF315324
<i>Melanosuchus niger</i>	JF315312

RESULTS AND DISCUSSION

Constructed tree showed different of *Osteolaemus tetraspis*, *Mecistops cathrapactus*, *T. schlegelii* and *Gavialis gangeticus* belonging in outgroup clade. The Neighbor-joining tree showed that every *Crocodylus* members of the Crocodylidae were monophyletic, while *O. tetraspis* and *M. cathrapactus* were have paraphyletic relationship with *Crocodylus* genus. The *T. schlegelii* and *G. gangeticus* have closest paraphyletic relationship with Alligatoridae. Whereas in UPGMA tree. *O. tetraspis*, *M. cathrapactus*, *T. schlegelii* and *G. gangeticus* placed paraphyletic with Alligatoridae. Within Alligatoridae, all members of Caimaninae have paraphyletic relationship with Alligatorinae. Without consideration of precision between UPGMA and neighbor-joining tree from *Cytochrome-b*, both trees shows contradiction with McAliley *et al.* (2006) and Meganathan *et al.* (2010). They use bayesian analyses, which used *Ornithine-decarboxylase* and *C-mos*, also *ND4* and *ND6* respectively. In their studies *O.s tetraspis*, *M. cathrapactus*, *T. schlegelii* and *G. gangeticus* are consistently placed within Crocodylidae clade.

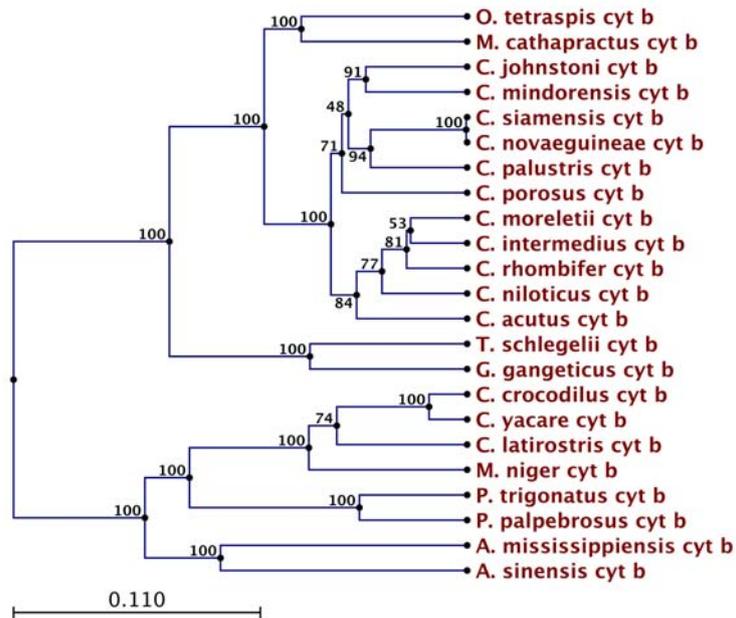


Figure 1. UPGMA tree obtained from *Cytochrome-b* analyses illustrating the relationship among species.

The primer we designed in this study showed a high specificity on species level. Only two primers for duo among genus of *Crocodylus* shares same primers (Table 2.). There is none for others eight in genus of *Crocodylus*. This also happened to in genus *Alligator*, *Caiman* and *Paleosuchus*. However, several Genus only have one member like *Mecistops*, *Osteolaemus*, *Tomistoma*, *Gavialis*, dan *Melanosuchus*. Their Species specific primers are consider as Genus specific primers with *Cytochrome-b*.

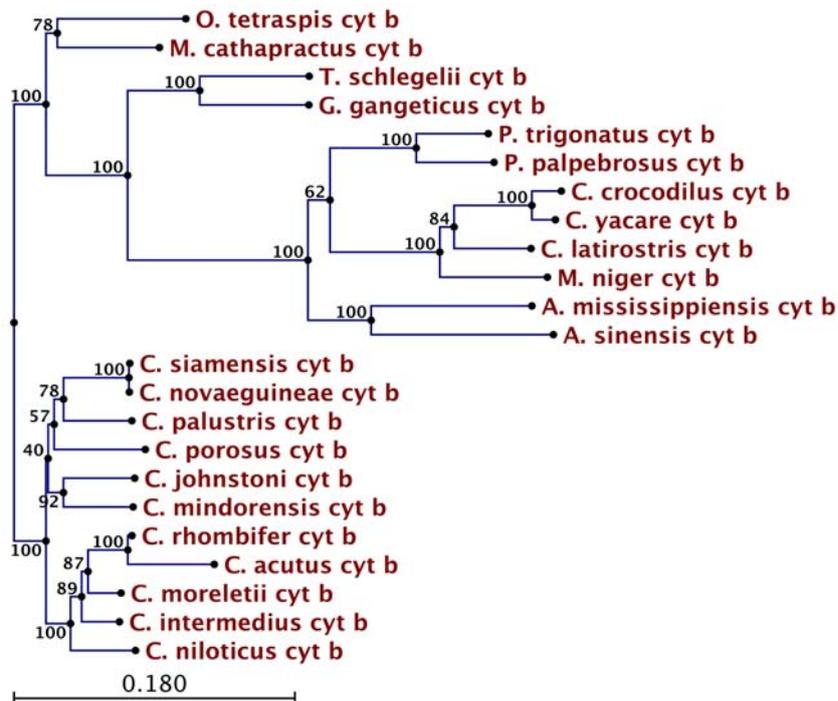


Figure 2. Neighbor-joining tree obtained from *Cytochrome-b* analyses illustrating the relationship among species.

Table 2. Results of designed primer from *Cytochrome-b*.

Species	Forward Primer (5'→3')	Reverse Primer (5'→3')	Product length
Genus <i>Crocodylus</i>* (12 Species)			
<i>Crocodylus novaeguineae</i>	'GCCGCTGTCGT	'GGAGTTGGGA	400
<i>Crocodylus siamensis</i>	CACACACCT'	CGGGGGCGT'	
<i>Crocodylus rhombifer</i>	'GGGCCATCAGT	'TGGGATAGAG	467
<i>Crocodylus acutus</i>	CAACAGCGCA'	GGCGCAGGCT'	
Genus <i>Mecistops</i>** (1 Species)			
Genus <i>Osteolaemus</i>** (1 Species)			
Genus <i>Tomistoma</i>** (1 Species)			
Genus <i>Gavialis</i>** (1 Species)			
Genus <i>Alligator</i>*** (2 Species)			
Genus <i>Caiman</i>*** (3 Species)			
Genus <i>Paleosuchus</i>*** (2 Species)			
Genus <i>Melanosuchus</i>** (1 Species)			

notes: *) 8 Species only have species specific primers, **) Species specific primers are considered Genus specific primers, ***) no Genus specific primers obtained

Specific primers may very valuable; moreover the genus specific primers are important for effective and efficient identification tools. Unanjak *et al.* (2011) and Meganathan *et al.* (2009) used the same principal: nested-PCR. Hence, in their studies, effectiveness of primers were tested in agarose gel. Although they do not accommodate all Crocodylian species and only showing Species specific, the primers already can distinguish DNA template target and showing no cross reaction. Their studies have a promising future for animal forensics and conservations. For this reason, we are still looking suitable loci to develop effective and efficient molecular identification tools.

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