# PHYLOGENETIC ANALYSIS OF FROGS IN KUMBE RIVER BASED ON COI GENE OF MITOCHONDRIAL DNA

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**Abstract**

The level of genetic variation and population originallity is important for directing conservation and managemen purposes. Thus we are conducting the research to determine and discuss phylogenetic relationship of common frog in Kumbe River, Papua and also to pointed the possible acts of conservation of such aquatic resources in a riverine system. The specimens in the form of larval for the study were obtained from Kumbe River System, Papua on the year of 2014. Total genomic DNA was extracted from whole larvae using a DNA extraction kit procedure and a partial fragment of the mitochondrial *COI* was amplified using universal primers Fish-COI-F and COI-Fish-R. The PCR products were visualised and sequencing reaction was performed by the EZ-Seq service (Macrogen) using the reverse primer (COI-Fish-R). Chromatograms were checked manually and multisequence alignments were done using MUSCLE. A neighbour-joining tree was constructed with the Kimura 2-parameter (K2P) model using MEGA version 5.0 software to infer the phylogeny of this species. The results reveal that PCR products of the *Cytochrome Oxidase Subunit I (COI)* mtDNA regions for the three common frog ponds samples produced fragments of 570 bp. DNA barcoding unable the species identification of larval morph types and the minimum genetic distance (0.171) or 82.9% similarities to the closes species. However the sequence of mitochondrial DNA gen COI has been successfully used in phylogeny analysis. The samples collected were identified only on genus level Rana sp, which has unique characteristic of nucleotide composition.

**Key words: Kumbe, phylogenetic, pond frog**

## Introduction

The genus *Rana* has been generated in two contexts, one is their confused taxonomy and other is the phylogenetic relationships among these species are still unresolved and constitute a basic cause of taxonomic confusion (Inger *et al.* 2007). Particularly on the larvae stage, morphological characters are only of limited use and are time consuming. Furthermore, larvae are so delicate that they are often badly damaged during collection, and fixation regularly causes the loss of pigmentation patterns important for identification (Smith, 1995). Different approaches, such as application of molecular markers, have now been commonly used to solve the problems in frog taxonomy.

DNA barcoding offer a unique technique to provide accurate, fast and automatable species identification by using short (usually initial 500bp) and standardized gene regions as internal species tags of gen Cytochrom oxidase subunit 1 (CO1) mtDNA (Packer *et al*. 2009; Hebert *et al*. 2003). Barcodes will aid the advance of fisheries science by providing a rapid, reliable system for the identification of specimens by non-taxonomists, regardless of the life stage (Valdez-Moreno *et al*. 2010). They also aid taxonomists by revealing overlooked diversity (Gregory, 2005) and speeding the description of new species by allowing specialists to concentrate in highlighted problems after the barcoding (e.g.see Quiroz-Va´zquez and Elı´as-Gutie´rrez, 2009). Aside from identification, barcode analysis is acceptance importance in life-history descriptions (Valdez-Moreno *et al.* 2010).

Lying at the collision line of the Australian and Pacific tectonic plates, Papua New Guinea is remarkably diverse in terms of landscapes, ecosystems, and species (Murdiyarso & Kurnianto, 2008). The number of frog species, is likely to double when all species have been discovered and scientifically named. Although the level of genetic variation and population originallity is important for directing conservation and management purposes, limited study has examined the

frog ponds species known from Papua river system due to enormous areas of the island. Thus we are conducting the research to determine and discuss phylogenetic relationship of common frog ponds in Kumbe River, Papua and also to pointed the possible acts of conservation of such aquatic resources in a riverine system.

**Material and methods**

*Area study, sample collection and preservation*

Sampling campaigns were conducted at Alfasera sampling site S'07'24'23.9' E'140'37'04.6' on May 2014 in Kumbe River, West New Guinea (Papua), (Figure. 1). Sampling and collecting representative samples of frog ponds larvae was carried out using two sets of 30 cm diameter modified bongo nets and the nets were maintained submerged around 5 cm from surface as long as 15 minutes. Samplings were performed early in the morning (06:00 – 07:00 A.M.) and late in the afternoon (05.00 – 06:00 P.M.). Immediately after larvae were caught, they were kept in water, manually sorted after collection and were subsequently individually stored in absolute ethanol of 1.5 mL.

Figure 1. Sampling sites of Alfasera in River Kumbe, Indonesia.

*DNA extraction, amplification and sequencing*

Total genomic DNA was extracted from muscle tissue of each specimen using the Extraction Kit procedure 'DNeasy Blood & Tissue' (Geneaid). A the partial fragment (501 bp) of mitochondrial Cytochrome C Oxidase Subunit-1gene (COI) was amplified using modified universal primers Fish-COI-F and COI-Fish-R described by (Ivanova *et al.* 2007): Fish-COI-F (5'- TAA TAC GAC TCA CTA TAG GGT TCT CCA CCA ACC ACA ARG AYA TYGG -3) and COI-Fish-R (5'-ATT AAC CCT CAC TAA AGG GCA CCT CAG GGT GTC CGA ARA AYC ARAA-3 ').

Amplification of the COI fragment was carried out in a 12.5 µL reaction volume consisting of 4.0 µL ultrapure water, 0.625 µL of each primer (1mM), 6.25 µL of 2 x QMP buffer (QIAGEN) and 1 µL DNA template (ca 100 ng/µL). PCR cycling parameters included an initial denaturing phase of 15 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 90 s at 55 °C and 30 s at 72 °C and ended with a final extension of 5 min at 72 °C. PCR products were visualized on a 1% agarose gel and purified using the A'SAP PCR clean-up kit (ArcticZymes). A sequencing reaction was carried out using the reverse primer (COI-Fish-R) by EZ-Seq service (Macrogen).

*Data analysis*

Chromatograms were checked manually, and multi-sequence alignments were done using MUSCLE (Dereeper, 2004). Additional sequences were obtained from BLAST searches of the NCBI Genbank database (http://www.ncbi.nlm.nih.gov/), and were used to identify specimens. A Neighbour-Joining tree was constructed with the K2P model with 100 bootstrap replications employing MEGA 5.0 (Tamura *et al.* 2013). Analyzed specimens were considered to belong to a specific taxonomic group only if they formed a cluster with a maximum of 3 % (K2P) sequences divergence (Hebert *et al.* 2003). Support for nodes in NJ analyses was assessed using non-parametric bootstrapping with 100 full heuristic pseudo-replicates. For comparative purposes,

we used the several sequences of the freshwater fish species in Genbank (Accession Number view in figure) to root the tree.

**Results and Discussion**

PCR products of the *Cytochrome Oxidase Subunit I (COI)* mtDNA regions for the three common frog ponds samples produced fragments of 570 bp (Figure. 2). DNA barcoding unable the species identification of larval morphotypes (Figure. 3). There are likely at least 130 frog species in the Papua. However the frog fauna remains poorly known and many of these frogs are likely to prove endemic to the island (Murdiyarso & Kurnianto, 2008). The non existing of the reference sequences of mtDNA COI lead to the inability the DNA barcode technique to clasify to species level. This indicates that relatively little biodiversity research has been performed on tropical peat swamp (Yule, 2010).

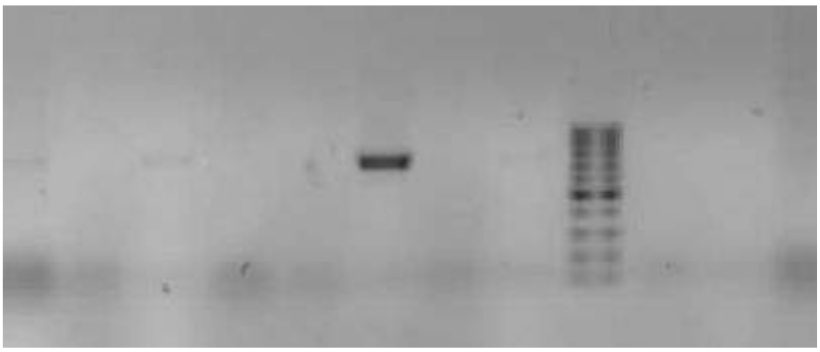

Figure 2. Sizes of amplified product of the COI region from the samples

Figure 3. Early life stages of common frog pons from peat swamps of the River Kumbe.

The final aligned data of COI yielded 570 characters comprising 570 conserved and no variable sites. The COI sequences were very rich in A + T content (Table 1), while the DNA profile shown in Figure. 4.

The NJ dendogram based on pairwise genetic distance indicated segregation of three larval morphotypes one cluster (Figure. 5). The NJ analysis and bootstrap estimates in this study showed minimum genetic distance (0.171) or 82.9% similarities to the closes species (Table 2), emphasizing their evolutionary relationship as well as divergence in recent times. This suggests the existence of a separate gene pool for these species. However the phylogenetic relationships among these species are still unresolved and constitute a basic cause of taxonomic confusion. Genetic distances are provided as heuristic measures of evolutionary isolation, but are not used as criteria for recognizing species (Inger *et al.* 2009).

Table 1 . Sequence characteristics of COI region of mtDNA in frog

| No | Parameters | *Rana sp from Kumbe System* |
|---|---|---|
| 1 | Aligned length (bp) | 570 |
| | No. of conserved sites | 570 |
| | No. of variable sites | 0 |
| 2 | G + C content (%) | 43.7 |
| | A + T content (%) | 56.3 |
| 3 | Nucleotide frequencies of | |
| | Adenine | 31.9 |
| | Thymine | 24.4 |
| | Cytosine | 17.7 |
| | Guanine | 26.0 |

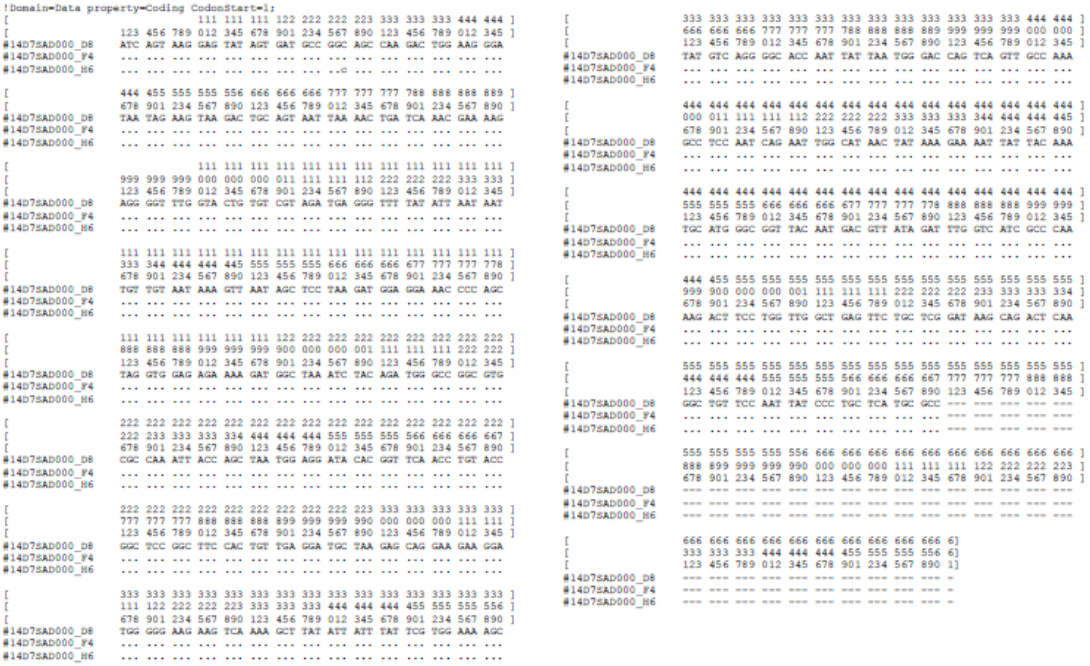

Figure 4. Sequence properties of Gen COI mtDNA from *Rana sp*

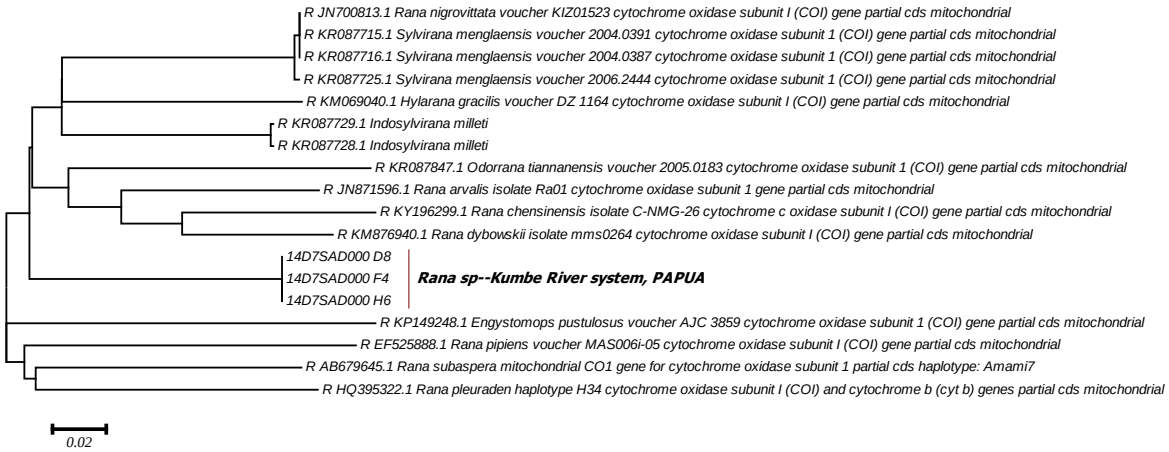


Figure 5. NJ dendogram constructed on the basis of genetic distance calculated from Kimura 2 Parameter among 3 frog species and its reference from NCBI

Tab. 3. Estimates of Evolutionary Divergence between Sequences of samples analysis

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 14D7SAD000_D8 | | | | | | | | | | | | | | | | | |
| 2 | 14D7SAD000_F4 | 0,000 | | | | | | | | | | | | | | | | |
| 3 | 14D7SAD000_H6 | 0,000 | 0,000 | | | | | | | | | | | | | | | |
| 4 | R_KR087729.1_Indosylvirana_milleti | 0,171 | 0,171 | 0,171 | | | | | | | | | | | | | | |
| 5 | R_KR087728.1_Indosylvirana_milleti | 0,171 | 0,171 | 0,171 | 0,002 | | | | | | | | | | | | | |
| 6 | R_KR087725.1_Sylvirana_menglaensis | 0,198 | 0,198 | 0,198 | 0,169 | 0,169 | | | | | | | | | | | | |
| 7 | R_KR087716.1_Sylvirana_menglaensis | 0,198 | 0,198 | 0,198 | 0,167 | 0,167 | 0,004 | | | | | | | | | | | |
| 8 | R_JN700813.1_Rana_nigrovittata | 0,198 | 0,198 | 0,198 | 0,167 | 0,167 | 0,004 | 0,000 | | | | | | | | | | |
| 9 | R_KR087715.1_Sylvirana_menglaensis | 0,198 | 0,198 | 0,198 | 0,167 | 0,167 | 0,004 | 0,000 | 0,000 | | | | | | | | | |
| 10 | R_KM069040.1_Hylarana_gracilis | 0,186 | 0,186 | 0,186 | 0,169 | 0,169 | 0,174 | 0,179 | 0,179 | 0,179 | | | | | | | | |
| 11 | R_KY196299.1_Rana_chensinensis | 0,223 | 0,223 | 0,223 | 0,229 | 0,229 | 0,228 | 0,233 | 0,233 | 0,233 | 0,256 | | | | | | | |
| 12 | R_JN871596.1_Rana_arvalis | 0,223 | 0,223 | 0,223 | 0,194 | 0,194 | 0,192 | 0,190 | 0,190 | 0,190 | 0,192 | 0,165 | | | | | | |
| 13 | R_KM876940.1_Rana_dybowskii | 0,215 | 0,215 | 0,215 | 0,194 | 0,194 | 0,204 | 0,209 | 0,209 | 0,209 | 0,248 | 0,128 | 0,155 | | | | | |
| 14 | R_AB679645.1_Rana_subaspera | 0,228 | 0,228 | 0,228 | 0,229 | 0,229 | 0,214 | 0,209 | 0,209 | 0,209 | 0,213 | 0,235 | 0,205 | 0,206 | | | | |
| 15 | R_HQ395322.1_Rana_pleuraden | 0,223 | 0,223 | 0,223 | 0,212 | 0,212 | 0,239 | 0,239 | 0,239 | 0,239 | 0,207 | 0,232 | 0,226 | 0,234 | 0,204 | | | |
| 16 | R_KR087847.1_Odorrana_tiannanensis | 0,215 | 0,215 | 0,215 | 0,225 | 0,225 | 0,222 | 0,225 | 0,225 | 0,225 | 0,224 | 0,222 | 0,214 | 0,208 | 0,240 | 0,259 | | |
| 17 | R_EF525888.1_Rana_pipiens | 0,233 | 0,233 | 0,233 | 0,240 | 0,240 | 0,246 | 0,244 | 0,244 | 0,244 | 0,237 | 0,238 | 0,251 | 0,252 | 0,235 | 0,224 | 0,256 | |
| 18 | R_KP149248.1_Engystomops_pustulosus | 0,220 | 0,220 | 0,220 | 0,240 | 0,240 | 0,243 | 0,243 | 0,243 | 0,243 | 0,259 | 0,277 | 0,292 | 0,260 | 0,261 | 0,241 | 0,291 | 0,266 |

Even though the result yield no proves that common frogs pond in the sampling site could classify to species level, the unique of sequences represent a species distinct from existing frog species, conservation efforts must be establish for this particular species. In the past, some conservation efforts were hindered by the uncertain identity of frog species, see leopard frogs. Resolution of Frog taxonomy within the ecosystem will allow for resources to be most effectively allocated to on the ground conservation activities (Golberg *et al.* 2004).

**Conclusions**

PCR products of the *Cytochrome Oxidase Subunit I (COI)* mtDNA regions for the three common frog ponds samples produced fragments of 570 bp. DNA barcoding unable the species identification of larval morph types and the minimum genetic distance (0.171) or 82.9% similarities to the closes species. However the sequence of mitochondrial DNA gen COI has been successfully used in phylogeny analysis. The samples collected were identified only on genus level *Rana sp*, which has unique characteristic of nucleotide composition.

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