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Molecular Barcoding and Phylogeny Reconstruction of Rhynchoporus sp in Minahasa North Sulawesi Based Partial Cytochrome Oxidase Sub Unit 1 Gene (CO1)

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Abstract
Molecular phylogeny reconstruction of *Rhynchophorus sp* from *Cocus nucifera* L, *Arenga pinata* and *Metroxylan sagu* was investigated using partial sequences of *Cytochrome c oxidase* subunit I gene (CO1). Three type of *Rhynchophorus sp* according the place of live used this study. *Rhynchophorus sp* from *Cocus nucifera* (AR1), *Metroxylan sagu* (SG1) and *Arenga pinata* (AR1) was analysis the partial CO1 gene. Phylogeny trees was constructed using MEGA 6,0 and Geneous 6,0. From the results of this studied, based on partial CO1 gene, *Rhynchoporus* living at the *Arenga pinata* is *Rhynchoporus palmarum* while *Rhynchoporus* living on Sago palm (*Metoxylan sagu)* and Coconut (*Cocos nucifera)* are *Rynchonporus vulneratus.* The results of this study is the first step of the revision of the uncertain taxonomic status and phylogenetic relationships among the *Rhynchophorus* genus in Minahasa, North Sulawesi. Further analysis should be performed using other *Rhynchophorus* species as well as other molecular markers.
Keywords: Rhynchophorus sp, CO1, Cocus nucifera, Arenga pinata, Metroxylon *sagu* Rottb, CO1, phylogeny tree

Intoduction

Based on the previous studies, the morphological characteristics *Rhychoporus sp* in Minahasa. has many variation according to their habitat (Korua, 2015). The number of morphological variation in *Rhychoporus sp* in Minahasa cause problems in the identification of species. Identification based on morphological characteristics may be less accurate in getting the position *Rhychoporus sp* species in Minahasa. *Rhychoporus sp* is an important insect species in Minahasa, North Sulawesi, because it can attack the main agricultural crop (Coconuts). Besides coconut, *Rhychoporus sp* are also found in plants such as Sagu (local name) and Aren (local name). From the results of studies conducted habitat, *Rhychoporus sp* on coconut plants can be moved to and Sagu based on the availability of food (Mokosuli, 2015). It is difficult to identify species *Rhychoporus sp* because of mixing populations increase intraspecies variation. Identification of the *Rynchoporus sp* that live in coconut, Aren and Sagu are important in the effort to control the population. Over population of *Rynchoporus sp* on thats plants had decrease the production. In addition, by knowing the position of the species, it is important to conserve *Rynchoporus sp* as a source of genetic diversity.

Identification of species using mitochondrial DNA gene as a barcode has become a tool for the identification of animal species around the world. Cytochrome oxidase subunit I (COI) mitochondrial gene was established as a bioidentification tool and has been used to study genetic variation in various insect species (Hebert et. al. 2003). Identification of species of insects in Sulawesi using barcode molecular DNA mitochondrial gene CO1 was performed on *Apis dorsata* Binghami and *Apis nigrocincta* Smith (Mokosuli et. al., 2013), Damselfly (Rantung et. al. 2015), Termites subteran (Ngangi et. al. 2015) and Marine insect Gerridae (Warouw et.al. 2016). Lodging in *Rhychoporus sp* identification using CO1 gene has been done on *Rhychoporus sp* living at Sago plant in Sorong and Raja Ampat Islands, Papua (Mokosuli et. al. 2015). In contrast to previous studies that analyze gene CO1 *Rhychoporus sp* that lived at Sago plants alone. The aims of this study was to get top notch and construction species phylogeny *Rhychoporus sp* that lived on plant Coconut, Aren and Sagu in Minahasa, North Sulawesi.

MATERIALS AND METHODS

Sample

Insects collecting used modified method Cheng *et. al.* (2010), by using neuston net. Collection on the fields area randomly. Insects that have been collected will insert in a bottle sample that has been labeled with place and time of data sampling. The bottle was filled with 95% alcohol for identification and preservation.

DNA Extraction, PCR Amplification and Sequencing

Total genomic DNA was extracted from *Rhychoporus sp* samples using Axygen Bioscience according to the manufacturer's protocol. PCR was performed in a total volume of 25 µL containing 1 × reaction buffer, 3 mM MgCl2, 0.24 mM dNTPs, 1.4 µM of each primer LCO1490 : 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 : 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et. al., 1994), 1U Go Taq Flexi DNA polymerase (Promega Corp.) and 2.5 µL of DNA (a 100 time dilution of the original DNA). The PCR program was as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 5 min. PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega Corp). Purified PCR products were analyzed by electro-phoresis in 1% agarose gel. The molecular size of the amplified products was estimated using 1 kbp DNA ladder (Biometra). PCR products were sequenced using AB1 PRISM Dye Terminator Cycle Sequencing Ready Reaction System, version 1.1. (Applied Biosystems) in FIRST BASE Singapura

Sequences Analyses and Phylogeny trees reconstructed

Obtained sequences were aligned using MEGA 6.0 and Geneous 6.0 software. Sequences were subjected to Basic Local Alignment Search Tool (BLAST) in order to perform sequence similarity searches (www.ncbi.nih.gov.com). Nucleotide frequencies were calculated using MEGA 6.0 software (Tamura et. al. 2013). The genetic distances (number of nucleotide substitutions per site) among sequences were calculated using the Maximum Composite Likelihood model in Geneous 6.0 software. Phylogenetic trees were reconstructed using two different reconstruction methods: (1) neighbor joining (NJ) and (2) maximum parsimony (MP). The NJ tree was reconstructed using the Maximum Composite Likelihood method. Phylogenetic analyses were conducted in MEGA 6.0 software. Bootstrap support values were obtained by 1,000 replications using both methods (Tamura et. al. 2013).

RESULTS AND DISCUSSION

DNA extraction used the tissue on hind legs of *Rhychoporus sp*. The results of PCR partial CO1 gene visualized by electogram of electrophoresis. Accordingly bands that formed showed the high concentration of amplicons partial CO1 gene in all sample (AR1, KL1 and SG1) (Figure 1).

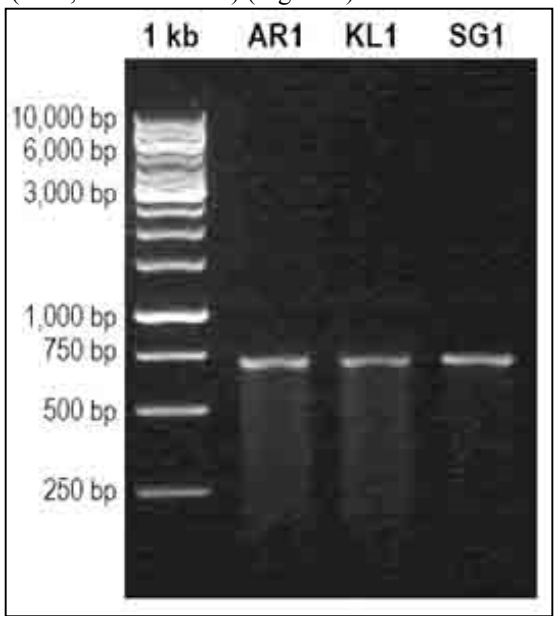


Figure 1. Visualization CO1 gene PCR amplicons by electrophoresis on 1% agarose gel . AR1 (Rynchoporus sp. From palm , coconut and KL1 of SG1 from Sagu) .

The sequencing results were interpreted using Geneous 6.0 software. The Molecular weight of partial CO1 gene sequences of KL1, AR1 and SG1 are 658 bp, 620 bp and 658 bp respectively (Gambar 2). Based of the chromatogram were resulted of the sequencing, showed all sequens of partial CO1 gene are good (Figure 2).

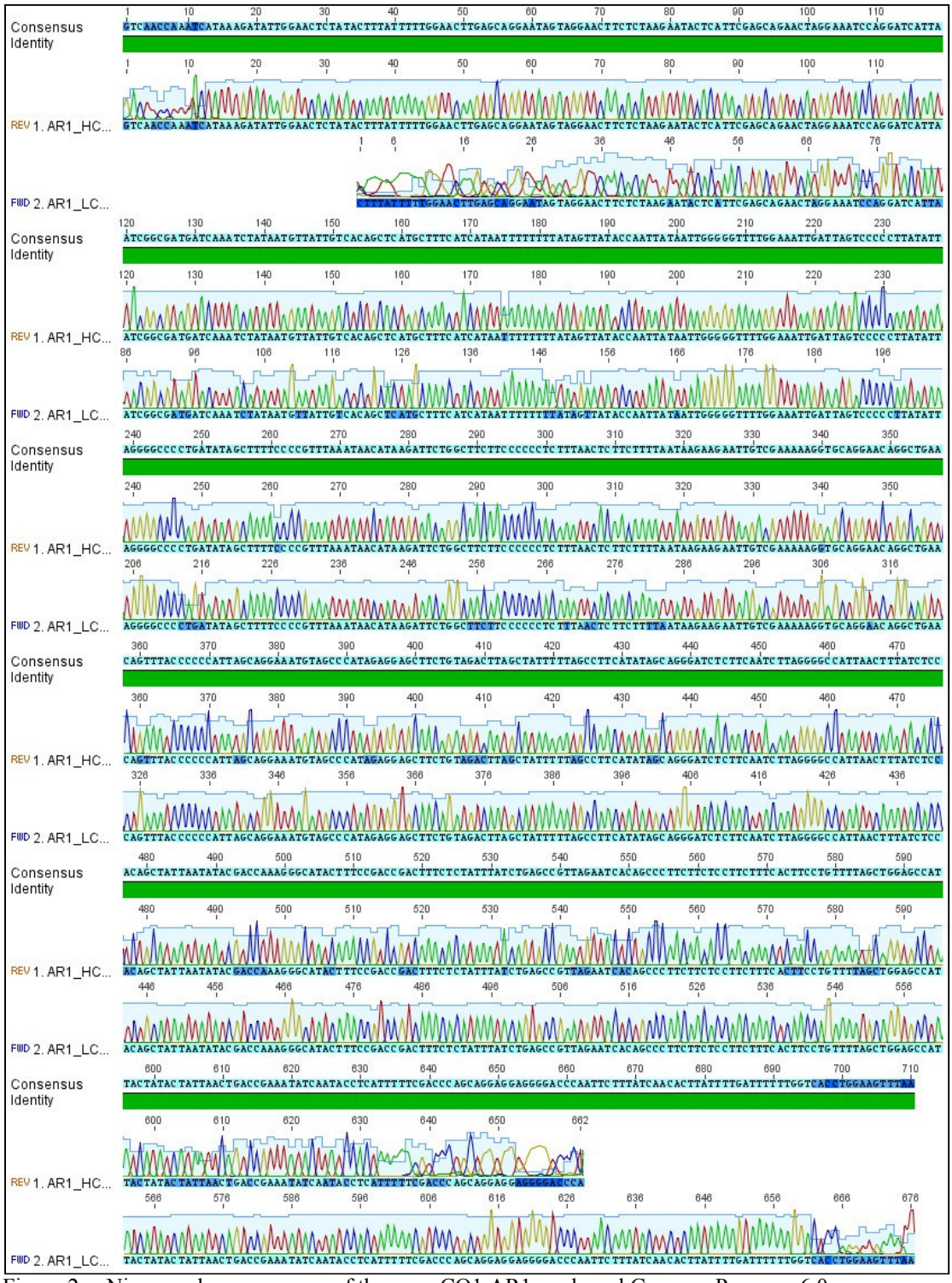


Figure 2a . Nigrogen base sequence of the gene CO1 AR1 read used Geneous Program 6.0

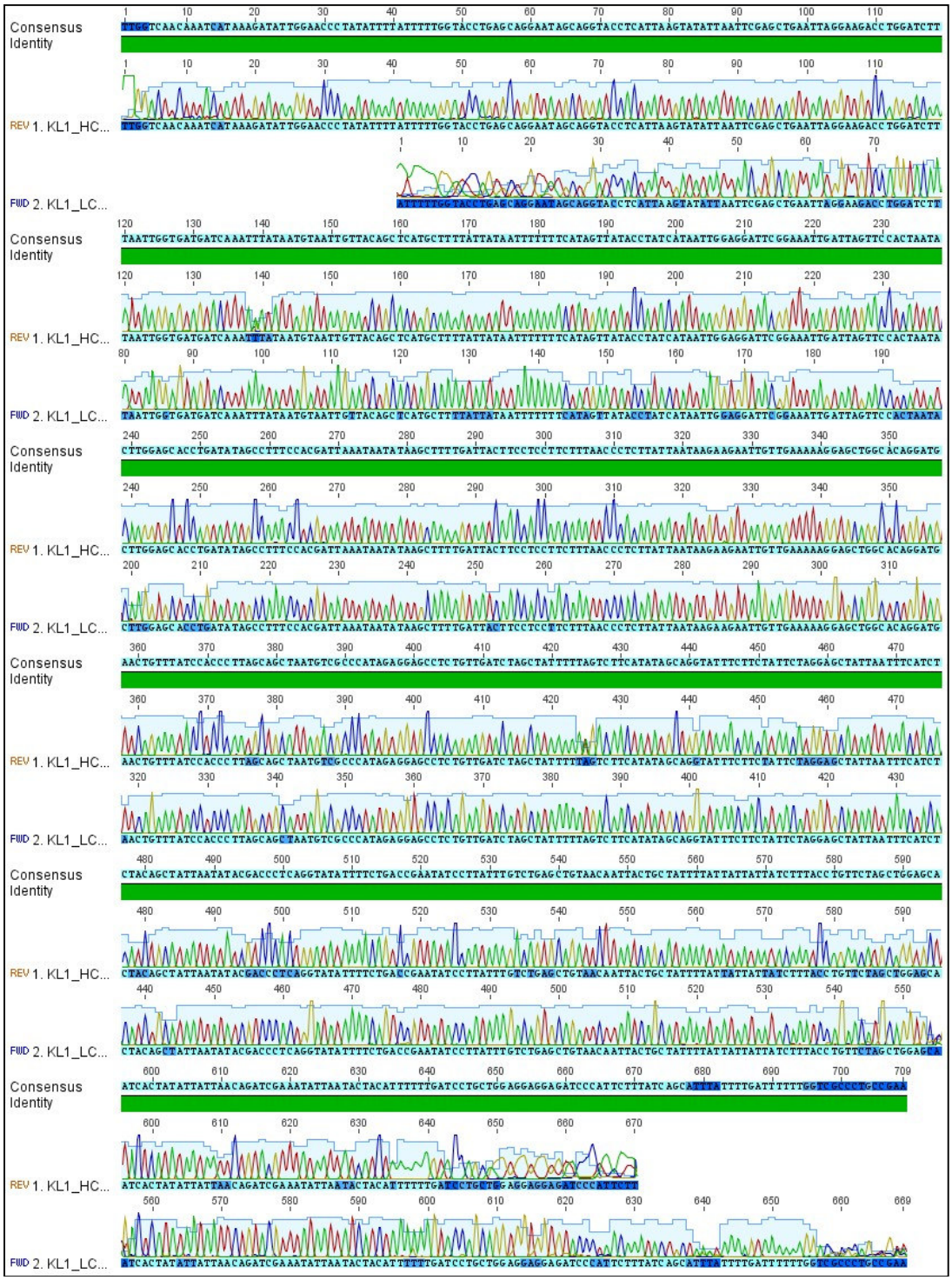


Figure 2b . Nigrogen base sequence of the gene CO1 KL1 read used Geneous Program 6.0

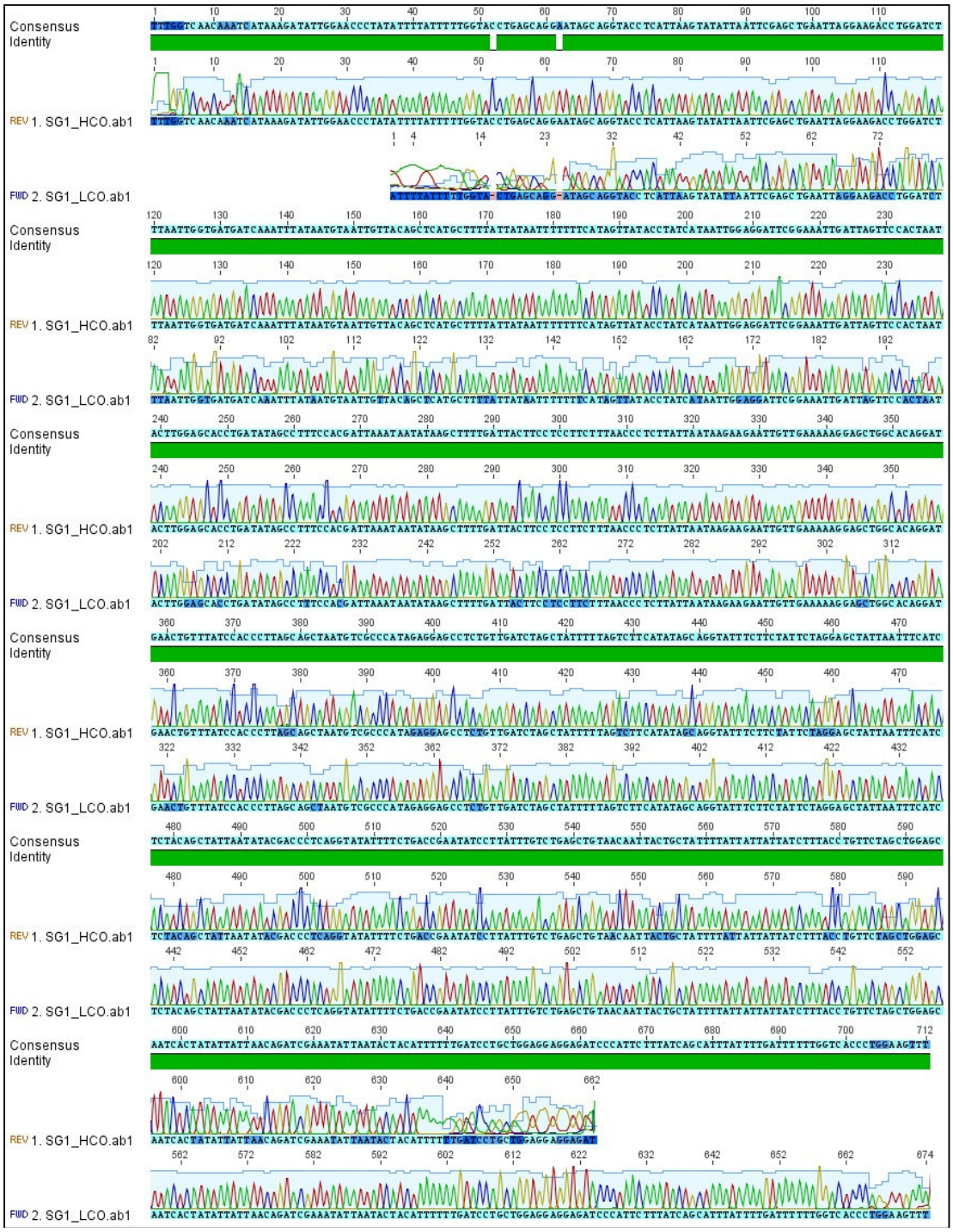


Figure 2c . Nigrogen base sequence of the gene CO1 SG1 read used Geneous Program 6.0

BLAST analysis results AR1 CO1 gene sequences showed the highest degree of similarity with *Rynchophorus vulneratus* accesion number [LN 612634.1][(Table 1). On the other hand partial CO1 gene sequences of KL1 and SG1 respectively showed the highest degree of similarity with *Rynchophorus cruentatus* accesion number [AY131113.1] (Table 2 and Table 3) .

Table 1 The percentage of sequence similarity AR1 CO1 gene sequences compared with the top ten recorded in the NCBI gene bank (www.ncbi.nih.gov/blast.com)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhynchophorus vulneratus mitochondrial partial COI gene for cytochrome oxidase subunit 1, isolate RV01A01	1024	1024	94%	0.0	98%	LN612634.1
Rhynchophorus vulneratus mitochondrial partial COI gene for cytochrome oxidase subunit 1, isolate RV01A02	1013	1013	96%	0.0	97%	LN612635.1
Rhynchophorus vulneratus mitochondrial partial COI gene for cytochrome oxidase subunit 1, isolate RV01A03	1000	1000	92%	0.0	98%	LN612636.1
Rhynchophorus vulneratus isolate RED1111 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	664	664	61%	0.0	98%	KF311631.1
Rhynchophorus vulneratus isolate RED939 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	664	664	61%	0.0	98%	KF311629.1
Rhynchophorus vulneratus isolate RED253 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	664	664	61%	0.0	98%	KF311567.1
Rhynchophorus vulneratus isolate RED1144 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	658	658	61%	0.0	98%	KF311633.1
Rhynchophorus vulneratus isolate RED935 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	658	658	61%	0.0	98%	KF311628.1
Rhynchophorus vulneratus isolate RED843 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	658	658	61%	0.0	98%	KF311621.1
Rhynchophorus vulneratus isolate RED823 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	658	658	61%	0.0	98%	KF311617.1

Tabel 2. The percentage of sequence similarity KL1 CO1 gene sequences compared with the top ten recorded in the NCBI gene bank (www.ncbi.nih.gov/blast.com)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhynchophorus cruentatus cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	693	693	99%	0.0	86%	AY131113.1
Dinoptera collaris voucher GBOL_Col_FK_6727 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	669	669	99%	0.0	85%	KM445325.1
Dinoptera collaris voucher BFB_Col_FK_9633 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	664	664	99%	0.0	85%	KM439730.1
Nosodendron fasciculare voucher BFB_Col_FK_8752 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	662	662	100%	0.0	85%	KM452239.1
Myrmechixenus vaporariorum voucher ZMUO<FIN>:000424 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	662	662	100%	0.0	85%	KJ961927.1
Pterostichus oristatus voucher GBOL_Col_FK_5123 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	658	658	100%	0.0	85%	KM444764.1
Protapion ruficrus voucher GBOL_Col_FK_3091 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	658	658	99%	0.0	85%	KM443977.1
Pterostichus oristatus voucher GBOL_Col_FK_4257 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	654	654	100%	0.0	85%	KM440434.1
Protapion ruficrus voucher BFB_Col_FK_9671 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	652	652	99%	0.0	85%	KM445949.1
Tachinidae gen. tachJanzen01 sp. Janzen01 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	652	652	99%	0.0	85%	JQ576361.1

Tabel 3. The percentage of sequence similarity SG1 CO1 gene sequences compared with the top ten recorded in the NCBI gene bank (www.ncbi.nih.gov/blast.com)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhynchophorus cruentatus cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	693	693	99%	0.0	86%	AY131113.1
Dinoptera collaris voucher GBOL_Col_FK_6727 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	669	669	99%	0.0	85%	KM445325.1
Dinoptera collaris voucher BFB_Col_FK_9633 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	664	664	99%	0.0	85%	KM439730.1
Nosodendron fasciculare voucher BFB_Col_FK_8752 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	662	662	100%	0.0	85%	KM452239.1
Myrmechixenus vaporariorum voucher ZMUO<FIN>:000424 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	662	662	100%	0.0	85%	KJ961927.1
Pterostichus cristatus voucher GBOL_Col_FK_5123 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	658	658	100%	0.0	85%	KM444764.1
Protapion ruficrus voucher GBOL_Col_FK_3091 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	658	658	99%	0.0	85%	KM443977.1
Pterostichus cristatus voucher GBOL_Col_FK_4257 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	654	654	100%	0.0	85%	KM440434.1
Protapion ruficrus voucher BFB_Col_FK_9671 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	652	652	99%	0.0	85%	KM445949.1
Tachinidae gen. tachJanzen01 sp. Janzen01 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	652	652	99%	0.0	85%	JQ576361.1

Analysis of Sequences partial CO1 gene

Analysis of transition and transversion (R = 0.72) with a model Maximum likelihood used MEGA 6,0; obtained frequency of nucleotides A = 25 % , T = 25 % , C = 25 % and 25 % Guanine. The forms of nucleotide substitution are shown in Table 4 .

Table 4. Nucleotides substitution form of partial CO1 gene of AR1 , KL1 and SG1

From\to	A	T	C	G
A	-	7.2727	7.2727	10.4546
T	7.2727	-	10.4546	7.2727
C	7.2727	10.4546	-	7.2727
G	10.4546	7.2727	7.2727	-

According to table 5, the similarity of *Rhynchoporus sp*. showed that KL1 had 19,5 % different with SG1 and AR1 respectively. SG1 and AR1 based of the genetic distance analysis are the same species (Table 5).

Table 5. **Genetic distances among nucleotide sequences from *Rhynchophorus* spp. based on the pairwise analysis of CO1 gene. sequences.**

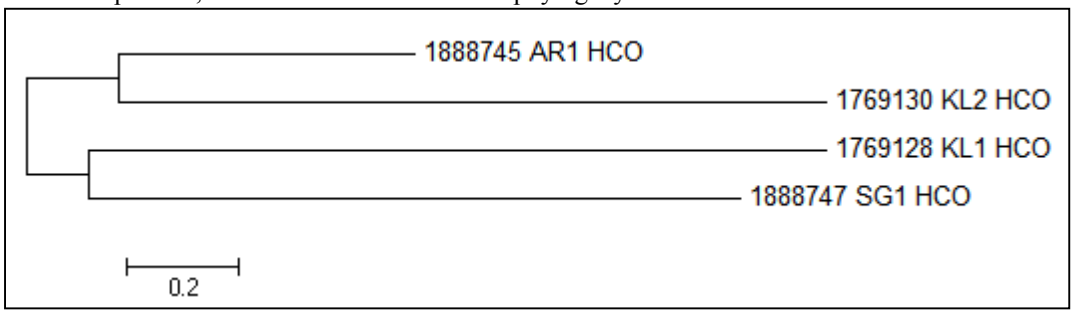
No.	Sample	1	2	3	4	5	6	7	8	9	10
1	AR1	100									
2	KL1	80.5	100								
3	SG1	80.5	100	100							
4	*Nosodendron fasciculare*	78.6	84.7	84.7	100						
5	*Dinoptera collaris*	79.5	84.7	84.7	85.4	100					
6	*Rhynchoporus palmarum*	82.2	81.8	81.8	81.7	80.8	100				
7	*Rhynchoporus bilineatus*	91.5	81.2	81.2	78.4	76.2	83.7	100			
8	*Rhynchoporus vulneratus* 1	97.7	78.4	78.4	75.7	74.7	82.1	92.4	100		
9	*Rhynchoporus vulneratus* 2	98	80.7	80.7	78.5	79.2	82.3	92.2	98	100	
10	*Rhynchoporus cruentatus*	79.7	81.2	81.2	76.5	78.3	85	85.1	85.3	84.9	100

Genetic variation in partial CO1 gene was supported by the results of previous studies, the morphometric analysis of KL1, AR1 dan SG1 were found some differences in morphometric characters among other forms of pronotum and color strip on the tip of the antenna on Rhynchoporus sp. thatch beige and Rhynchoporus sp. the black palm (Korua et al, 2015). Polimorfisme can occur in a population when more than one morphological variations at the same location and time (Ford, 1965, Abad et. Al. 2014). In case of random mating and every individual has the potential to mate, then morphological changes can take place in a population (Abad et. Al. 2014). Research conducted at Rhynchoporus *Rhynchoporus ferrugineus* Oliver and *Rhynchoporus schach* in the central and southern Philippines found that poliymorhism were major factor morphology modifications of the *Rhynchoporus sp*. Morphological modifications are also found in *Rhynchoporus phonicis* in Cameroon (Abad et. al., 2014; Tambe *et. al.* 2013).

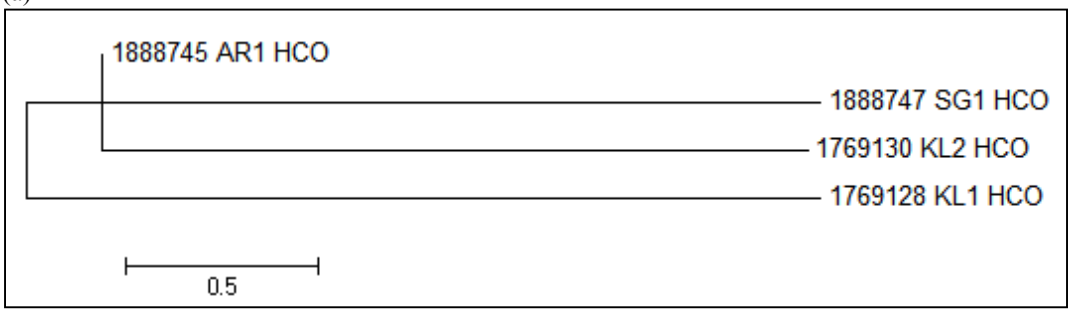
In previous studies conducted by the author had founded morphological modifications of imago *Rhynchoporus sp*. which livet on a palm tree, palm and sago palm in Minahasa, North Sulawesi Province (Korua, 2015). The results of this study imply that *Rhynchoporus sp*. has a high ability to adapt in their environment.

Reconstruction of Phylogenetic tree based partial COI gene.

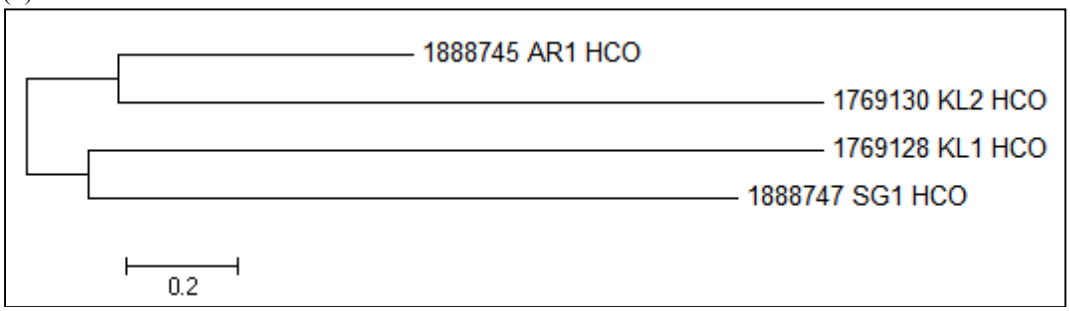
Nucleotide sequences were used to construct this tree. Construction phylogeny performed using three models namely Maximum Likelihood (ML), Neighbor-Joining (NJ) and Minimum Evolution (ME) (Tamura et. al., 2013, Kimura, 1980) (Figure 3). Three models are used to determine whether there differences in phylogenetic relationships AR1, KL1 and SG1 when the tree phylogeny constructed with different models.



(a)



(b)



(c)

Figure 3. Comparison of Construction Phylogeny CO1 sequences AR1 , KL1 and CO1 CO1 SG1 with three types of models (a) ME , (b) ML and (c) NJ

Reconstruction of the phylogeny tree was also performed using BLAST sequence results at www.ncbi.nih.gov/blast.com. Phylogeny tree constructed using the Neighbor Joining method using Geneous 6.0 program. KL1 and SG1 form one node but still be monophyletic with *Rhynchoporus palmarum* and *Rhynchoporus cruentatus*. But the phylogeny tree had formed, showed KL1 and SG1 closer relationship with *Rhynchoporus palmarum*. Different from KL1 and SG1, partial CO1 gene of AR1 had formed node with *Rynchophorus*

vulneratus (Figure 4).

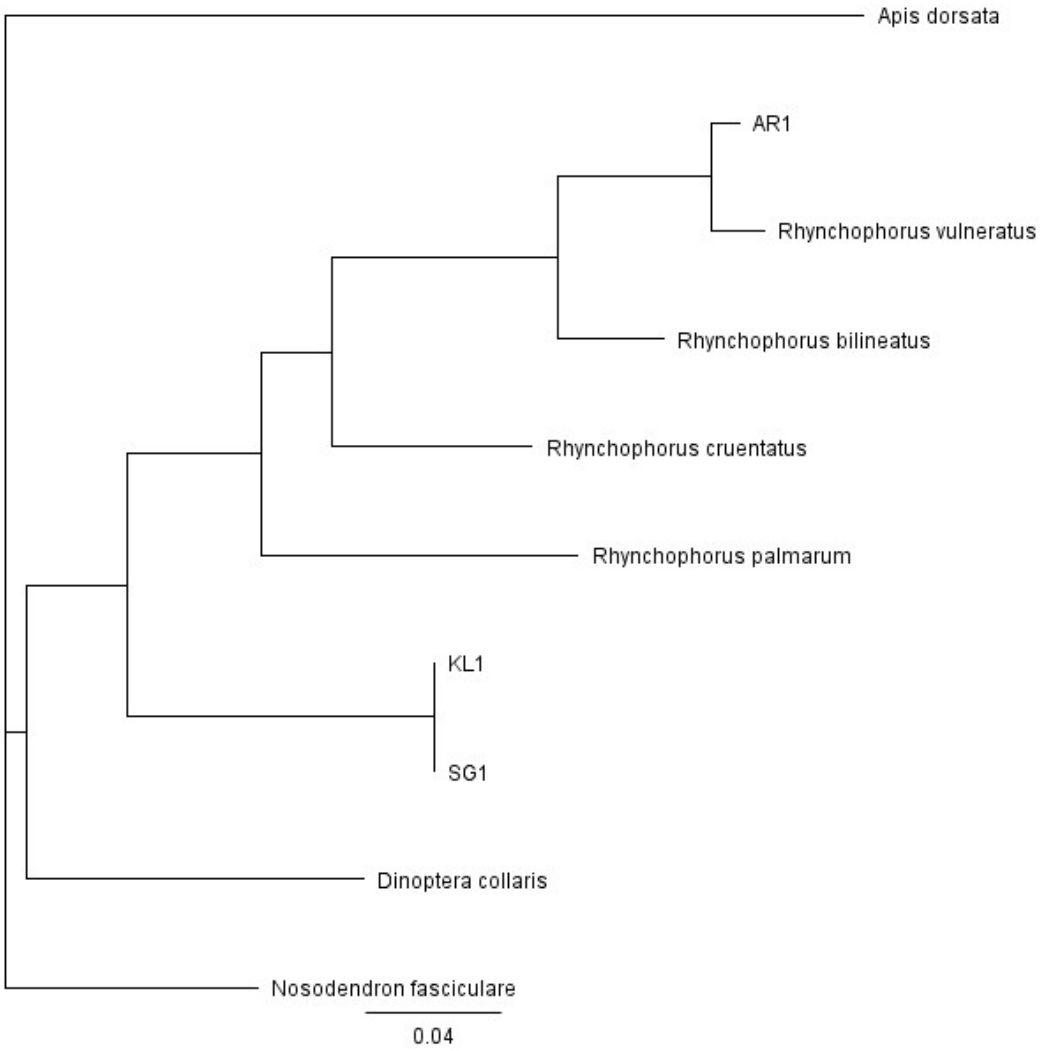


Figure 4. Tree phylogeny Rhynchoporus sp . (KL1 = Coconut , SG1 = Rumbia 1 , AR1 = Aren) .

CONCLUSIONS

From the results of the present study, based on partial CO1 gene, note that *Rhynchoporus* living at the *Arenga pinata* is *Rhynchoporus palmarum* while living on Sago palm (*Metoxylan sagu)* and Coconut (*Cocos nucifera)* are *Rynchonporus vulneratus* .

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