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Characteristics of the cytochrome oxidase c subunit 1 gene, cockroaches from the hospital

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Abstract
Cockroaches are members of insects that are most easily found in residential areas. Geographic isolation has given rise to new species of Cockroaches in various continents. Cockroaches have the potential to transmit more than 64 pathogenic microbes to humans. Research has been carried out which aims to obtain the position of species and the genetic characteristics of Cockroaches based on the cytochrome oxidase c subunit 1 (CO1) gene. Cockroach samples were obtained from the government general hospital Prof. dr. Kandouw, Manado, North Sulawesi, Indonesia. Genomic DNA extraction uses tissue in cockroach legs. Amplification of the CO1 gene uses universal primer forward LCO 1490 gene: 5 'GGTCAACAAATC ATAAAGATATGG3' and Reverse HCO 2198: 5'TAAACTTCAGGGTGACCAAAAAATCA3', carried out by PCR and vizualization of amplicons using 0.8% agarose electrophoresis. Sequencing has carried out at Singapore's First BASE Laboratory. Based on the results of BLAST and the construction of cockroach CO1 gene phylogeny, it has the closest resemblance to *Periplaneta americana*. The sample MRKE sequence has mutated at 14 nucleotide sites and has a genetic distance of 2.7% with five other sequences. The other five sequences do not have different genetic distances and nucleotide sites.

Keywords: COX1, cockroaches, hospital, north Sulawesi

Introduction
Cockroaches are members of insects that are most easily found in residential areas. Based on fossil evidence, Cockroaches have lived on earth 300 million years ago (Beccaloni and Eggleton 2013) [4]. Cockroaches are one of the most successful species of insect adaptation to all types of climate on earth (Robinson, 2005) [18]. In addition, Cockroaches become one of the insects that can live and adapt to the human environment. Geographic isolation has given rise to new species of Cockroaches in various continents. It is estimated that there are around 3500 species of Cockroaches, but only five species that affect humans. The five species are Periplaneta americana (American Cockroaches), Blattella germanica (German Cockroaches), Autralasiae periplaneta (Australian Cockroaches), Blatta orientalis (Oriental / Asian Cockroaches), Supella Longipalpa (Robinson, 2005; Chamavit *et al*. 2011; Wu and Lee, 2005; Beccaloni and Eggleton 2013) [18, 5, 26, 4].
The average body length of Cockroaches ranges from 10-50 mm, the body is oval, flat, long and active at night. Cockroaches have legs that are a lot of thorns and can run fast. Cockroaches are omnivorous insects but prefer food sources such as sweets, cheese, meat products and derivatives, starch and oil (Beccaloni and Eggleton 2013) [4]. However, Cockroaches also eat plants, vegetables, and fruit. Cockroaches like a warm and moist environment with abundant food. Cockroaches are also found in alkaline and moist ditches (Jirage, 2011). Cockroaches also eat faces of humans and other animals. In cockroach mouth organs there are glands that produce special chemical substances by leaving a smell on the food. Cockroaches can secrete allergens. Periplaneta americana produced three allergen compounds with molecular weights 26 - 79 kDa while Blatodea germanica produced 6 allergen compounds with molecular weights of 18-90 kDa (Wu and Lee, 2005) [26]. With these characteristics, the hospital can be an ideal habitat for Cockroaches. Cockroaches are often used as test organisms in integrated pest management (IPM). In addition, Cockroaches are also used as test organisms in biochemical, neurobiological and animal behavior studies (NCSU, 2019) [15].
Cockroaches can be vectors of various types of parasites. Cockroaches serve as intermediary hosts for several bacterial species, namely: Salmonella, Staphylococcus, Streptococcus, Escherichia coli, Proteus, Klebsiella, Serratia and some protozoa such as Giardia, Balantidium, Entamoeba histolyca, Trichomonas; mushrooms like Aspergillus. It was further reported that cockroaches coexisted with around 150 species of bacteria, 60 species of fungi, six species of yeast, 90 species of protozoa, 45 species of pathogenic ringworms and several other worms (Pai *et al*. 2003, Miso *et al*. 2005, Salehzadeh *et al*. 2007, Sookrung *et al*. 2008, Karimizarchi and Vatani 2009, Fakoo-rziba *et al*. 2010, Akbari *et al*., 2014) [17, 12, 20, 21, 10, 6, 1].
Research conducted by Chamavit *et al*., (2011) [5] by isolating Cockroaches from traditional markets, from 920 adult Cockroaches, protozoan and worm parasites were obtained in the abdomen by 54.1%. Scientifically proven, Cockroaches can transmit or become microbial pathogens that cause diarrhea, dysentery, cholera, leprosy, plague, typhus and viral diseases (WHO, 2018). Cockroaches are

classified by the United States Food Drug Administration (FDA) as a species of insect with a large health risk (Olsen *et al*. 2001; Sulaiman et.al. 2011) [16, 22].

Some species of Cockroaches can live in the same habitat. Preliminary studies conducted at the Government General Hospital, Prof. dr. Kandouw Malalayang Manado found several species of Cockroaches based on morphology. Knowing the position of species is very important information for overcoming the cockroach population. Moreover, Cockroaches from the hospital can become mechanical carriers and vectors of transmission of various types of diseases caused by pathogenic microbes.

Cockroaches included in the Blattoidea Order have "intelligence" in intraspecies communication using receptors on the antenna. Cockroach antennas have a long size, so there are many chemical receptors (Beccaloni and Eggleton, 2013) [4]. About 30 species of Cockroaches are associated with human habitat. As an undesirable insect, many cockroach eradication efforts are carried out. The use of insecticides has triggered morphological and physiological adaptations. Morphological adaptation is driven by molecular genetic adaptation at the level of genes and DNA. Kim and Rust, 2013 reported a change in the dominance of cockroach species in Asia and America. It is suspected that more adaptive species occur in a habitat. This is in line with the principle of evolution, where adaptive species will continue to survive while less adaptive species will be marginalized. Morphological modification can obscure cockroach identification based on morphological parameters. Therefore identification of cockroach species must combine morphological approach and molecular genetic approach. Molecular identification can complement the results of morphological identification. Identification of cockroach morphology from Prof. dr. Kandouw hospital found more than one species. Whether Cockroaches that have morphological differences are still in one species or have different species, need to be studied using a molecular approach. Molecular identification reported online in the gene bank, can complement data on insect species throughout the world, making it a reference for researchers around the world.

The cytochrome oxidase subunit 1 (CO1) gene from mitochondrial DNA is very sensitive in identifying insects. Sequences of CO1 genes with a length of 600 bp - 700 bp in mitochondrial DNA have been accepted as barcodes of species that are practical and standard for animals (www.barcoding.si.edu; Hebert *et al*. 2003; Savolainen *et al*. 2005; Ratnasingham and Hebert, 2007) [8]. The evolution of the CO1 gene occurs slowly compared to other protein-coding mitochondrial genes and is widely used to estimate molecular phylogeny. Therefore the CO1 gene is used as a tool in modern systems for population studies and evolution of animal species (Holingsworth, *et al*., 2011; Karimian *et al*., 2014; Mokosuli et.al. 2013) [11, 13]. The CO1 gene has been proven to be accurate in identifying several disease vector insects in North Sulawesi, including in *Anopheles* sp. (Manuahe *et al*. 2015) [14]; fruit fly (Sumampouw *et al*. 2017) [23]; *Aedes* sp. (Timah and Mokosuli, 2017) [23, 25], *Musca* sp. (Rotty *et al*., 2018; Kanan and Tulung, 2017) [19]. Identification of cockroaches from various habitats at Prof. dr. Kandouw hospitals Manado has been done using the CO1 gene. The results of molecular identification are used to obtain species position and the relationship of cockroach phylogeny.

Materials and Method

Samples Collection

A total of 10 adults Cockroaches were used as tissue sources for genomic DNA extraction. Samples of Cockroaches from the hospital after being captured were preserved with 100 ml of 95% ethanol in a sample bottle. After 1 x 24 hours, the cockroach sample was transferred to a new bottle containing 100 ml of 95% ethanol. Furthermore was used for DNA analysis.

Genomic DNA Extraction

Extraction and purification of mtDNA Cockroaches using the Genomic DNA Mini KIT (Tissue) Geneaid procedure. Stages of DNA extraction namely Lisis, Binding, Washing, and Elution. The initial stage before entering the extraction of mtDNA is tissue dissociation which consists of taking 30 mg of roach foot tissue and entering it in a 1.5 ml microcentrifuge tube. Then 200 µl GT Buffers and 20 µl proteinase K. Then incubated for 2 x 24 hours at 560C. Lysis stage: 200 µl GBT buffer was added then vortex for 5 seconds and incubated for 20 minutes at 60 ° C (shake tube every 5 minutes during incubation). At this stage, the elution buffer is placed on the thermoblock at 60 ° C to be used at the elution stage. Stage Binding: 200 µl of ethanol is added and shake for 10 seconds. The GD Column is then inserted into the 2 ml collection tube. Using a micropipette, the mixture was transferred to GD Column and then centrifuged at 13,200 rpm for 2 minutes. Move the GD Column to the new collection tube. Washing Phase: 400 mL W1 is added to this stage The buffer is then centrifuged at 13,200 rpm for 30 seconds. Move the GD Column to the new collection tube, adding 600µl of the wash buffer then centrifuged at 13,200 rpm for 30 seconds. Move the GD column to the new collection tube then centrifuge at 13,200 rpm for 3 minutes. Stage Elution: move the GD column to the new collection tube. Add 100µl elution buffer, on the GD column right in the middle of the matrix column, leave it at room temperature for 5 minutes. Centrifuge at 13,200 rpm for 30 seconds, then transfer the solution in the collection tube to the 100 µl tube. Then coded for Cockroaches DNA. The results of cockroach genomic DNA extraction became the template for the CO1 gene amplification stage.

a. Analysis of DNA purity and concentration

The results of genomic DNA extraction were then analyzed for concentration and purity using implant nanophotometer. DNA purity can be seen with an A260 / A280 ratio between 1.8 - 2.0 nm. If <1.8 means that many contaminants are in the form of proteins and/or other components of contamination of protein derivatives, and if> 2.0 means that they are contaminated with RNA (Kit Protocol).

b. Amplification of CO1 and Electrophoresis Genes

PCR was used 2x MyTaq HS Red Mix Bioline and primary universal CO1 gene. The primary CO1 gene consists of forward LCO 1490: 5'GGTCAACAAATCATAAAGA TATTGG3 'and Reverse HCO 2198: 5'TAAACTTCA GGGTGACCAAAAAATCA3' (Folmer et.al. 1994). Amplification was performed using Cyler Rotor-Gene Qiagen. The components and conditions of PCR applied can be seen in the following table. The PCR

component used is 2x MyTaq HS Red Mix Bioline 25 µL, LCO primer 1 µL, HCO primer 1 µL, 2 µL cockroaches DNA template and 21 µL ddH2O. While the PCR

conditions applied are shown in table 1. The PCR process was performed using a Qiagen Gene Rotor. Visualization of the CO1 gene amplicon from the PCR was carried out using 0.8% agarose electrophoresis.

Table 1: PCR Conditions

Cycle	Duration (Seconds)	Temperatur (°C)	Phase
35 x	60	94	Denaturation
	30	50	Annealing
	30	72	Extension
	60	72	Final Extension

c. Sequencing and analysis

Sequencing uses ABI PRISM 3730xl Genetic Analyzer develop by Applied Biosystems, USA through Singapore FIRST BASE sequencing services. The sequencing output in the form of a seq file was analyzed using Geneious 10.1.1. The reading results use Geneious 10.1.1. is a sequence chromatogram and base sequence of COI cockroach genes. The COI roach gene sequences obtained were then used for alignment analysis using the BLAST (Basics Local Alignment Searching Tools) method on the NCBI website (www.ncbi.gov). Selected sequences of BLAST results are then used to reconstruct the phylogeny tree. Reconstruction of phylogeny trees using MEGA 7.0. The phylogeny tree model used was determined by substitution analysis to obtain a suitable model based on the CO1 gene sequence obtained. Based on the results of the substitution analysis, the construction of the phylogeny tree uses the Neighbor-Joining method with the 1000x bootstrap. The construction of phylogeny trees was also done online on the NCBI website.

Results and Discussion

Cockroach genomic DNA extraction was successfully carried out using Genomic DNA Mini KIT (Tissue) Geneaid. The highest total DNA concentration was 35 µg / µl, while the lowest was at 25.35 µg / µl. The lowest total DNA purity was 1.67 while the highest was 1.85 (A260 / A280). The extracted roach DNA works well as a template for the amplification of the CO1 gene. This is proven by amplicons visualized through electrophoresis electromagnetic bands. The bands of each sample were visualized at 600 bp - 700 bp (Figure 1). Bands that were formed were not smeared so that it can be concluded that cockroach mitochondrial DNA is well extracted. However, the conditions and components of the PCR used are optimal.

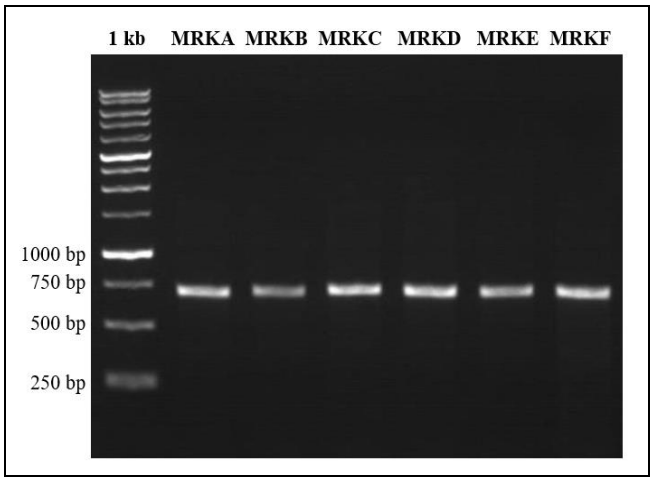
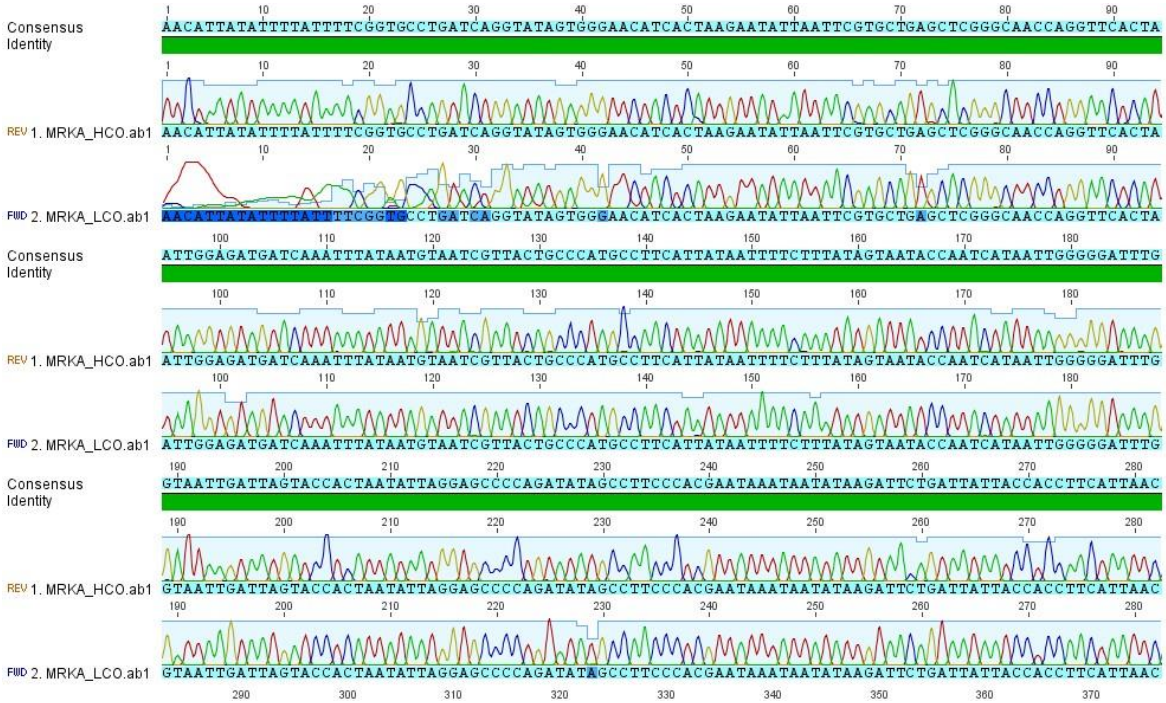


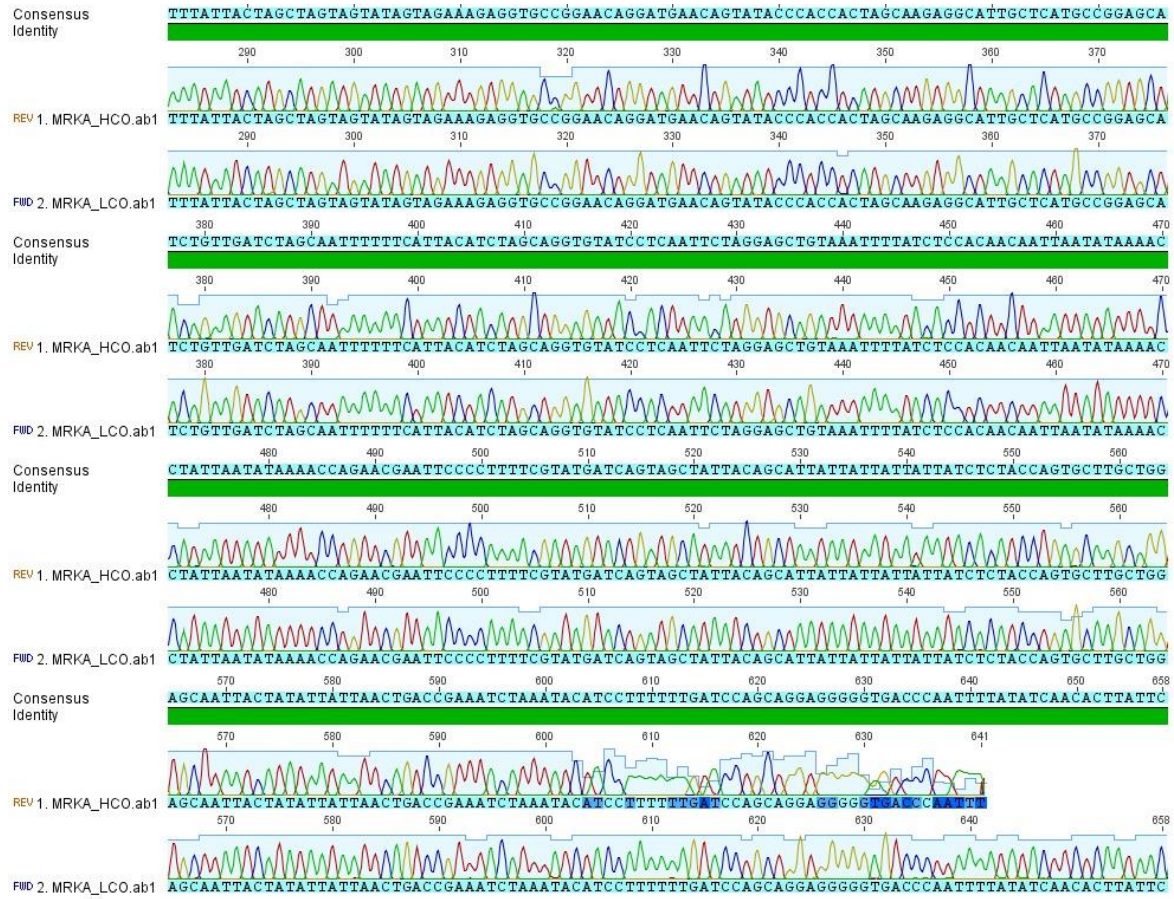
Fig 1: Amplicon CO1 gene from cockroaches, visualized using 0.8% agarose electrophoresis, DNA ladder each well 0.2µg, 1µl volume, ladder DNA 1 kb.

Sequencing

Sequencing produces good chromatogram. This is indicated by each band representing a type of nucleotide, not coinciding with each other (Figure 2). The forward sequence and reverse CO1 gene are then aligned using Geneous to obtain the consensus area of the CO1 gene from cockroaches (Figure 2).'

>MRKA

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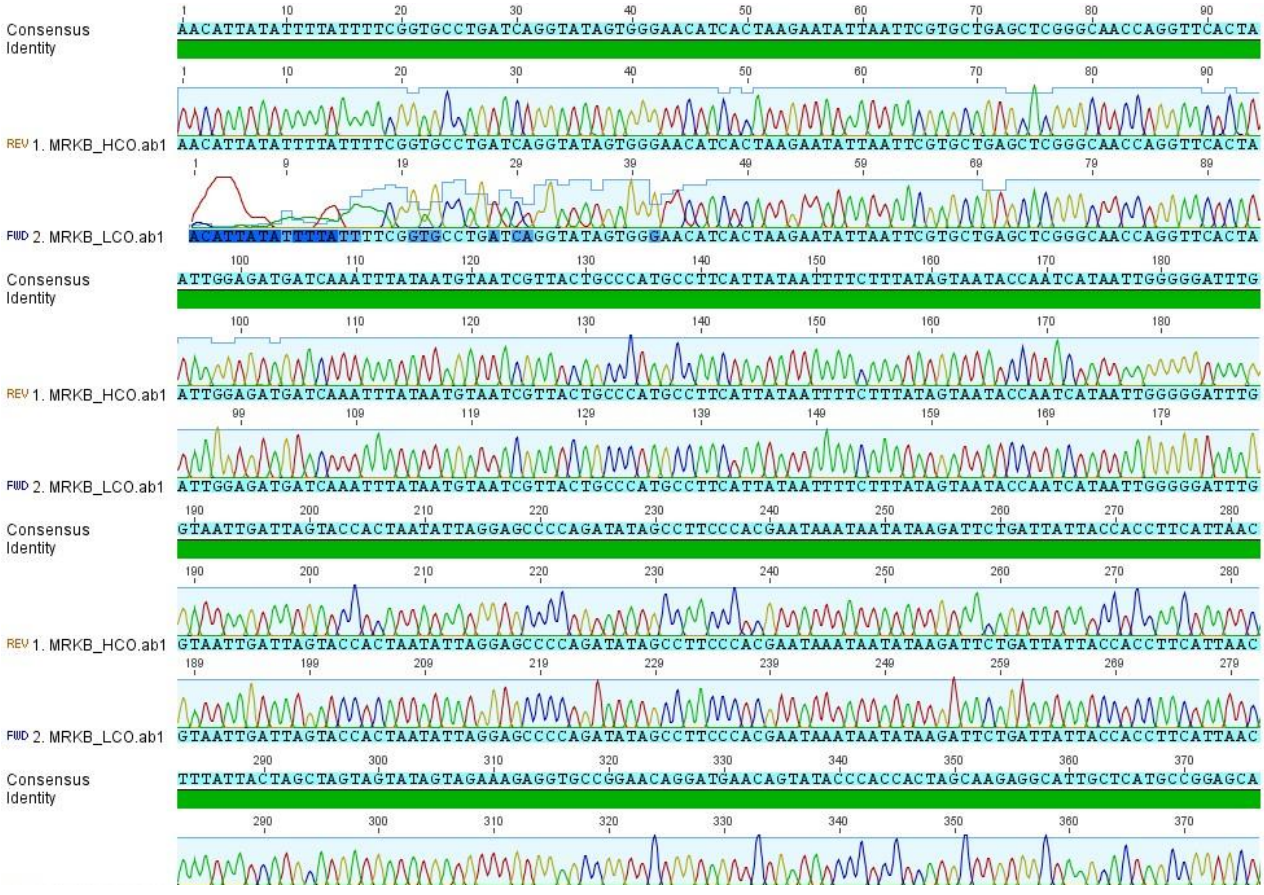
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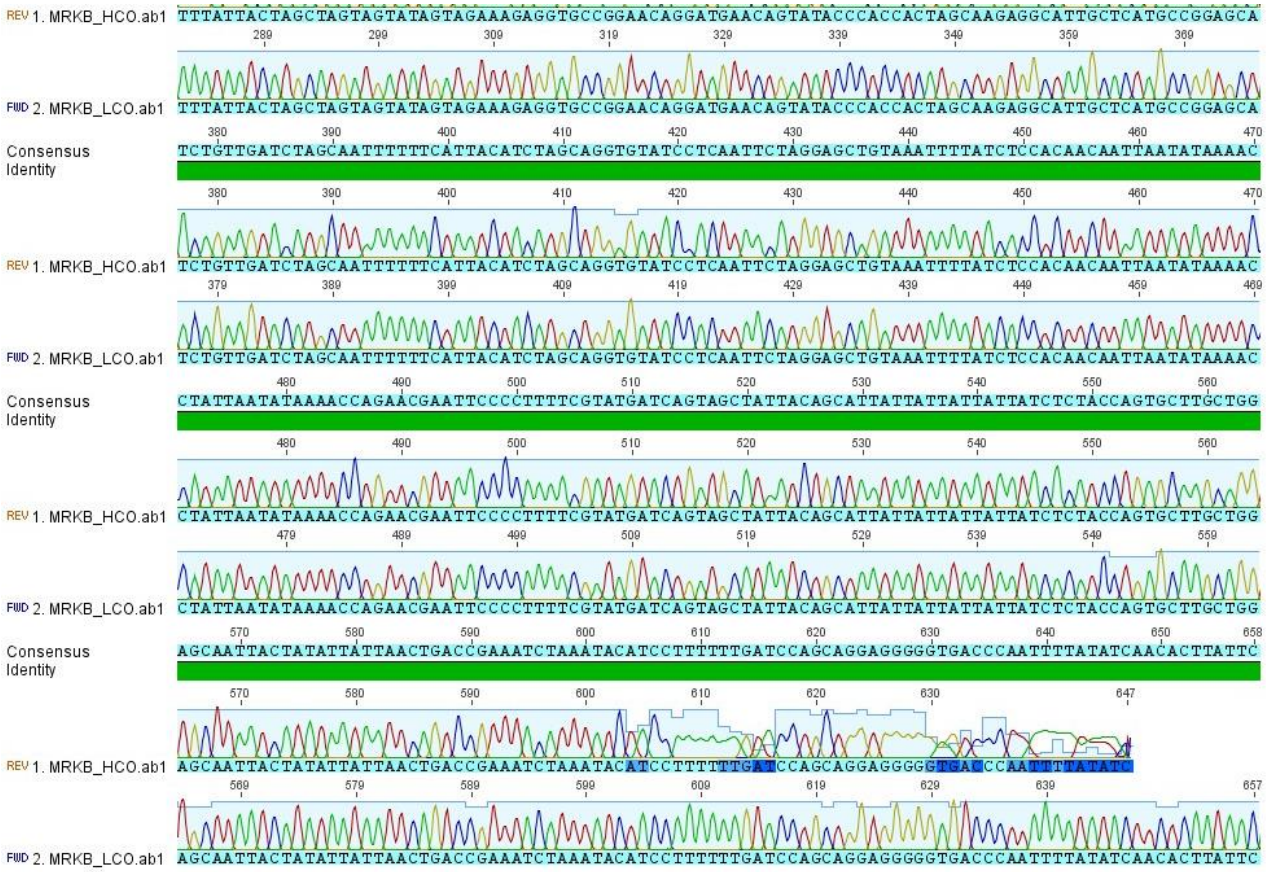
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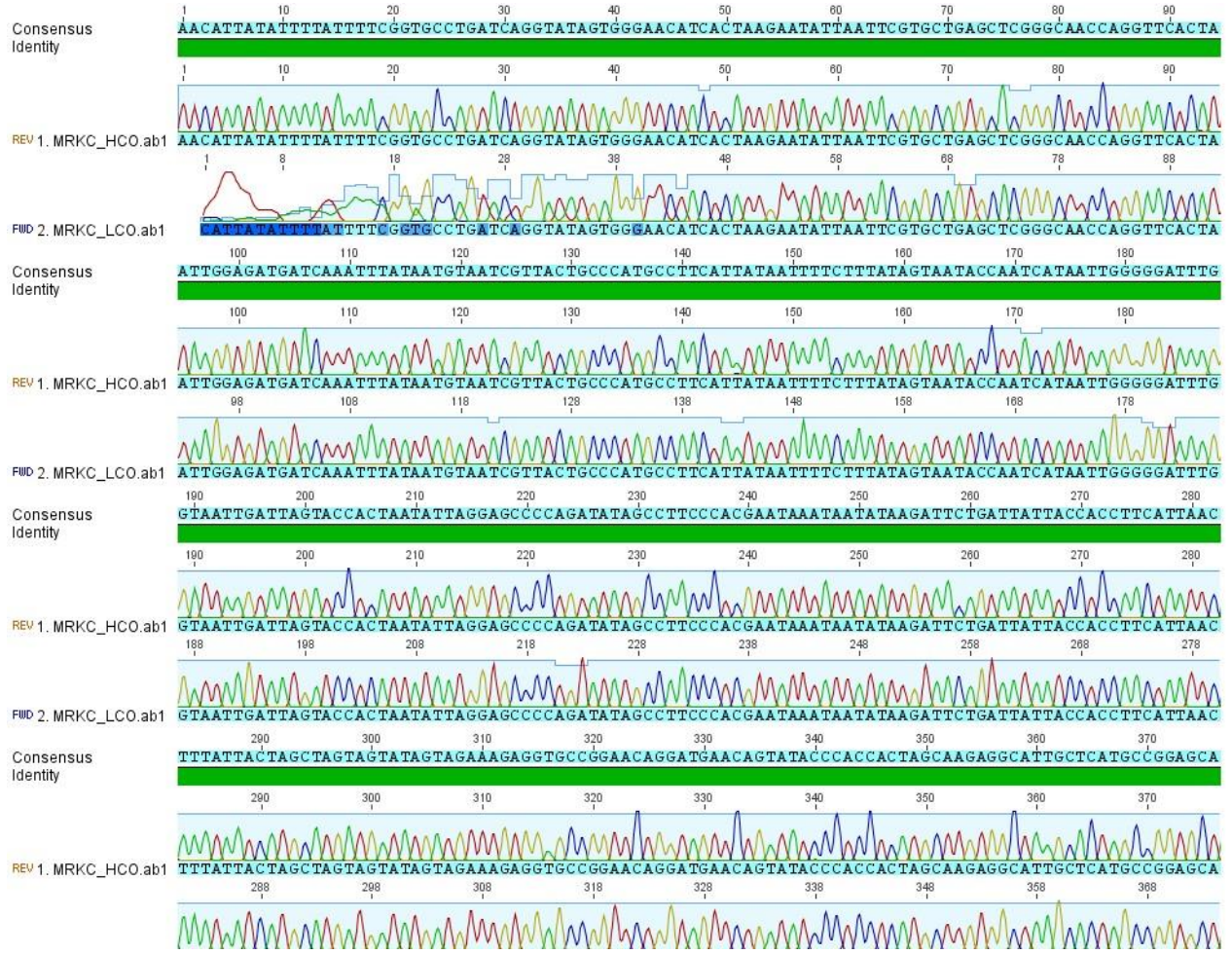
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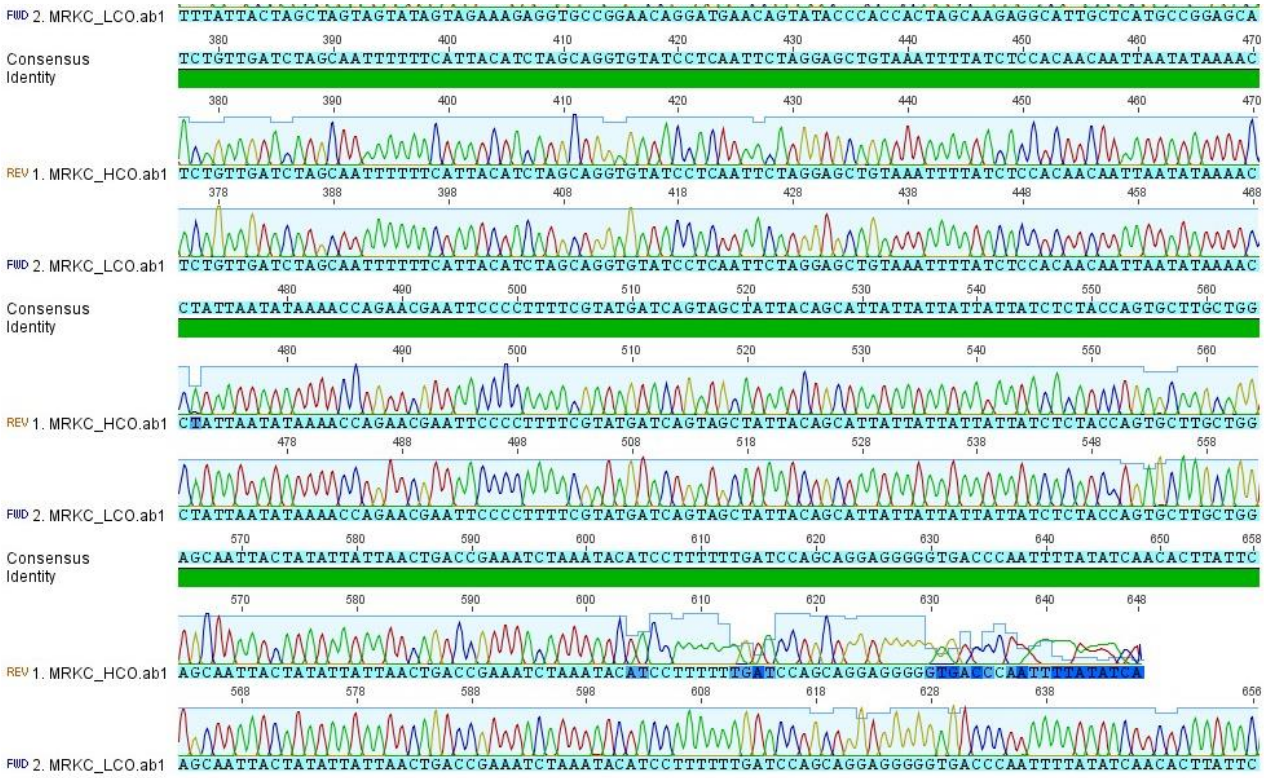
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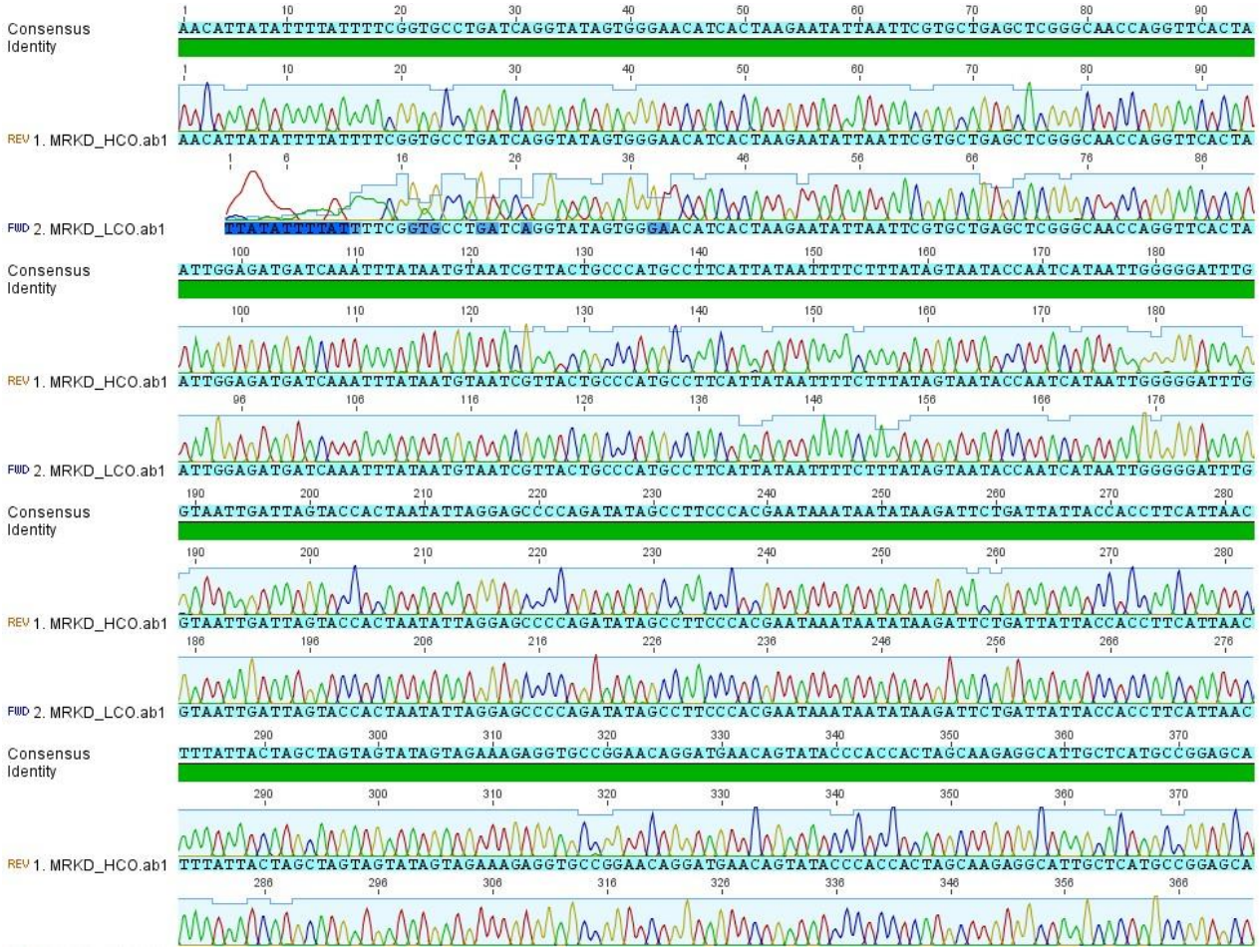
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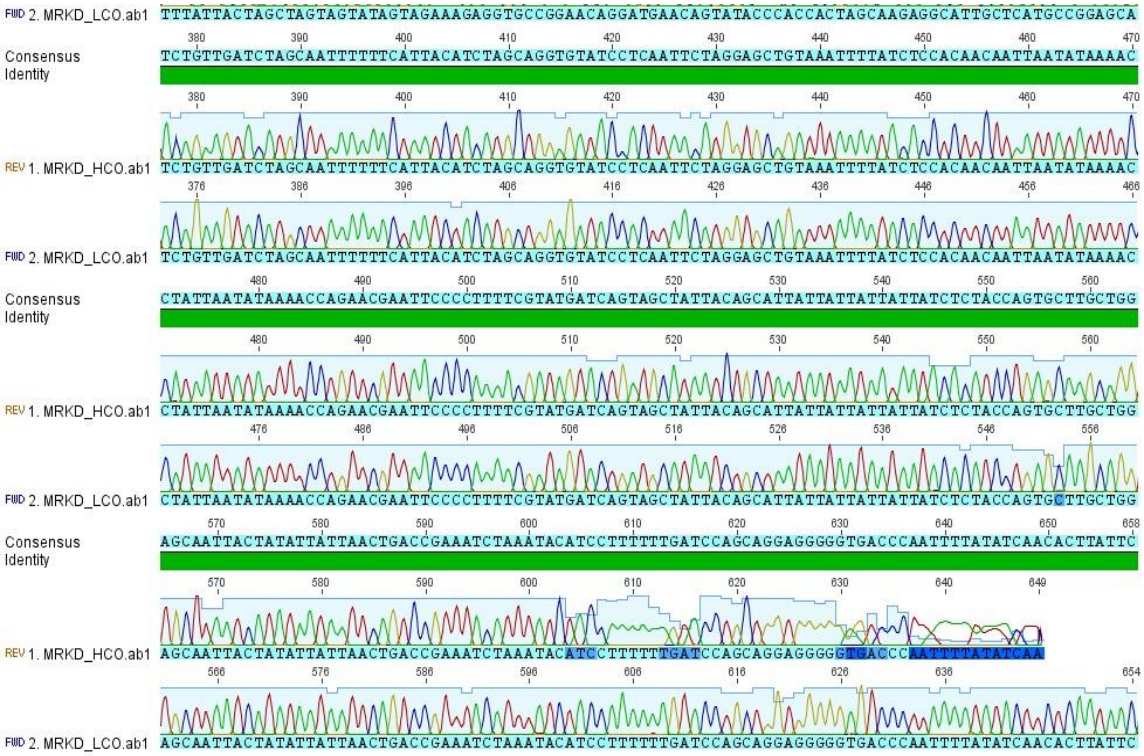
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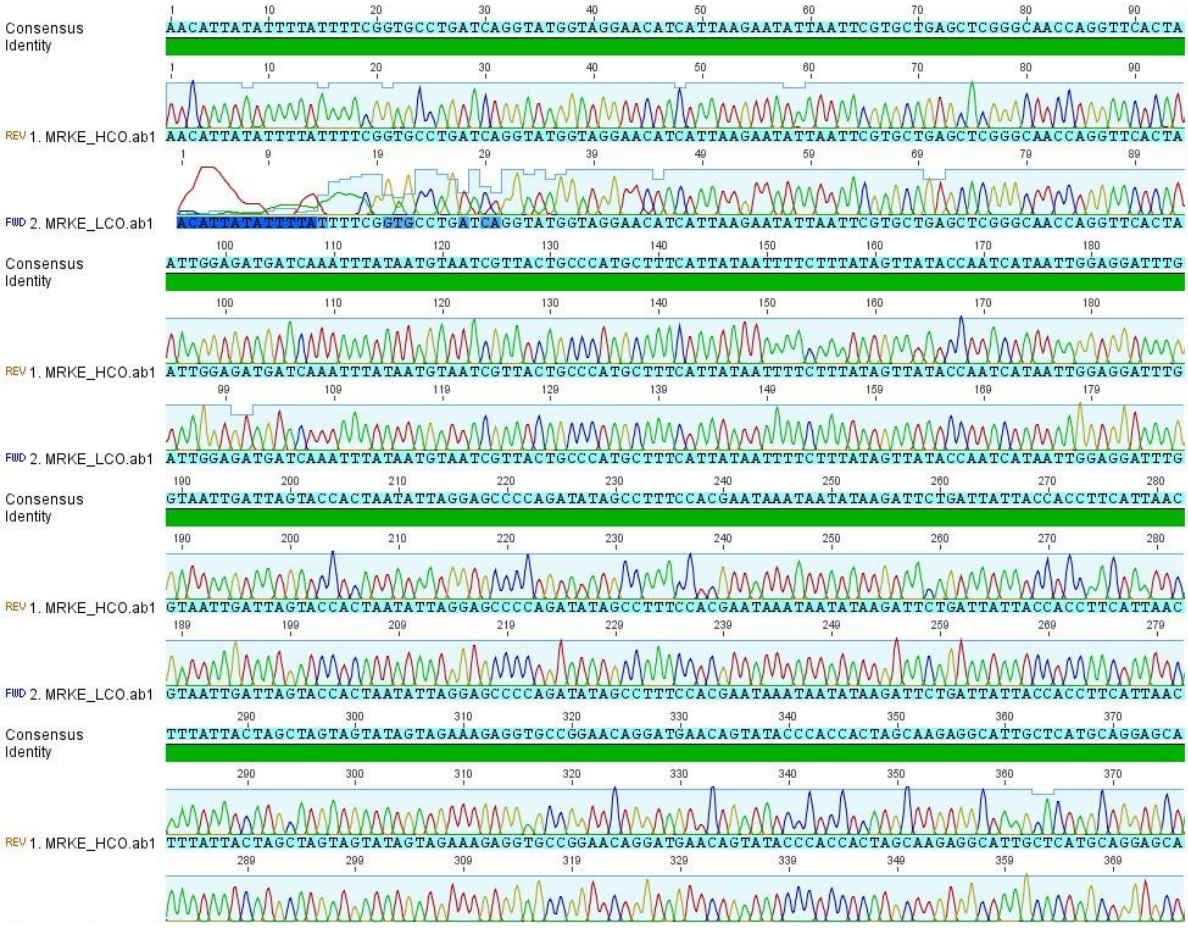
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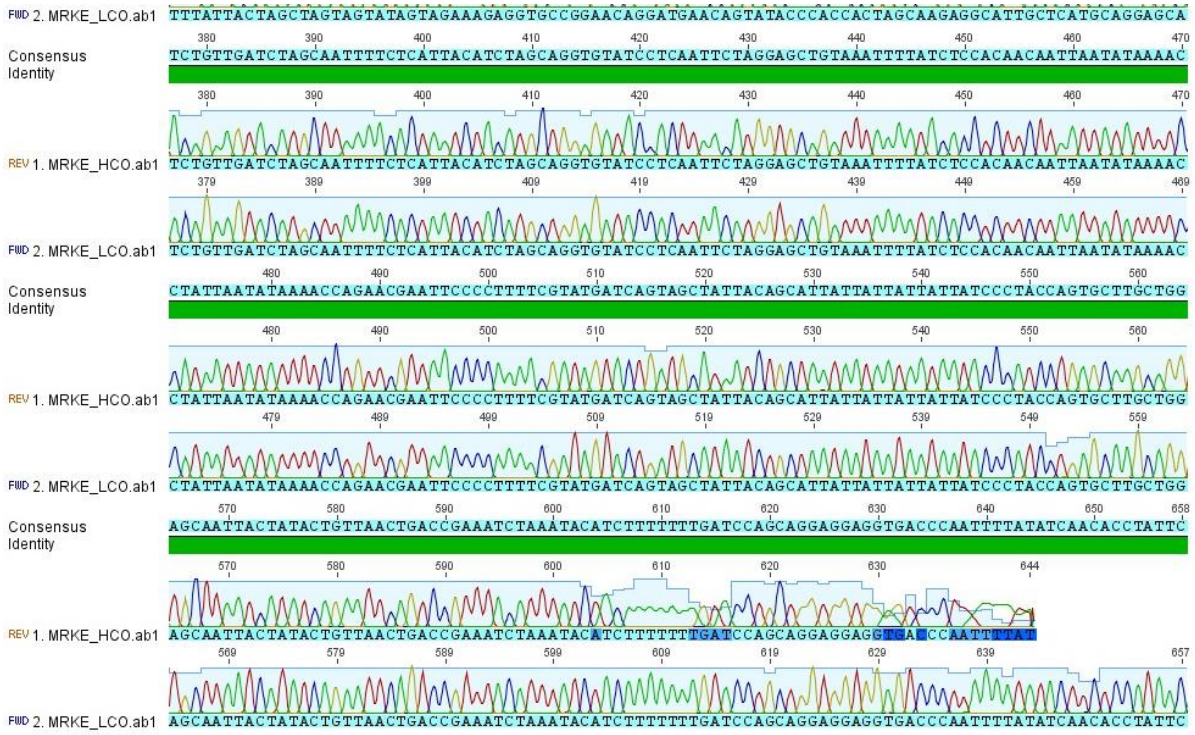
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Consensus
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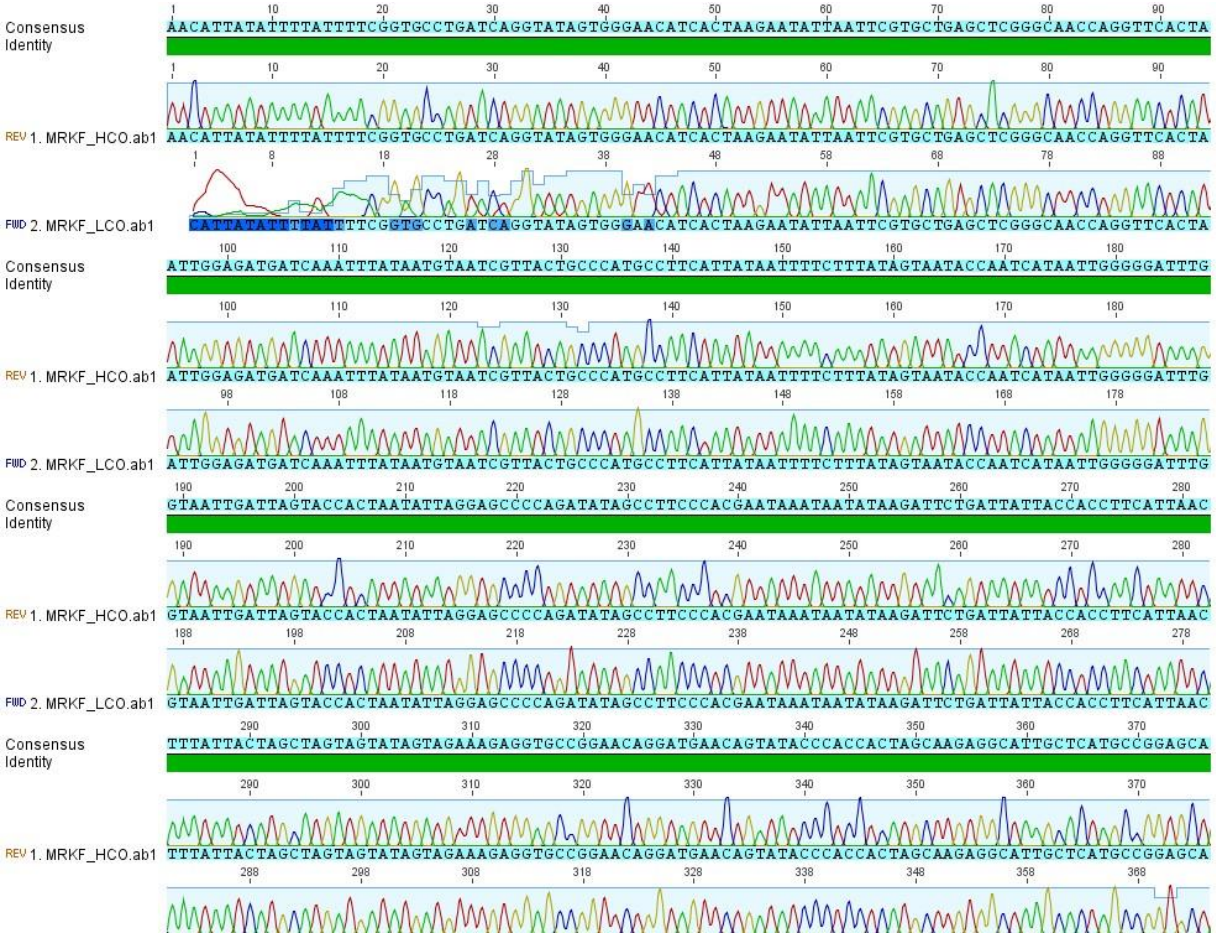
REV 1. MRKE_HCO.ab1
FWD 2. MRKE_LCO.ab1

>MRKF


Consensus
Identity
REV 1. MRKF_HCO.ab1
FWD 2. MRKF_LCO.ab1
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Identity
REV 1. MRKF_HCO.ab1

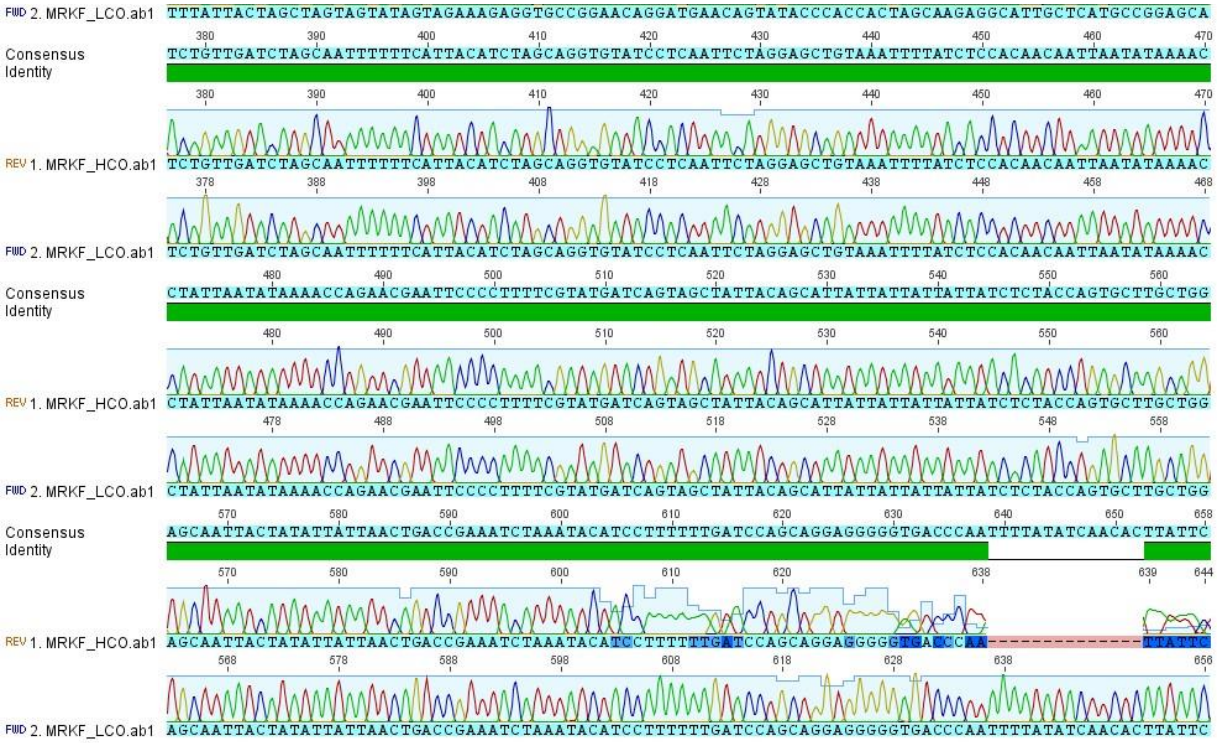


Fig 2: Chromatogram characteristics of CO1 cockroach gene sequencing results.

The composition of nucleotides and amino acids

Analysis of the composition of the nucleotide of the CO1 gene was carried out using MEGA 7.0. Of the six samples of the CO1 gene, only the MRKE sample has a different composition of Thymine, Cytosine, Adenine, and Guanine. The MRKA, MRKB, MRKC, MRKD, and MRKF samples have the same nucleotide composition.

Table 2: Nucleotide composition of the cockroach CO1 gene from RSUP Manado

	T(U)	C	A	G	Total
MRKA	33,0	19,0	31,9	16,1	658,0
MRKB	33,0	19,0	31,9	16,1	658,0
MRKC	33,0	19,0	31,9	16,1	658,0
MRKD	33,0	19,0	31,9	16,1	658,0
MRKE	33,1	18,8	32,1	16,0	658,0
MRKF	33,0	19,0	31,9	16,1	658,0

Ileusine and serine were the most encoded amino acids in the cockroach CO1 gene. While the amino acids asparagine and glutamine were amino acids that are not found in the CO1 cockroach gene (Appendix 3). The CO1 MRKE gene encodes the number of amino acids that were different from other samples. Valine amino acids were only coded by MRKE samples (Figure 3).

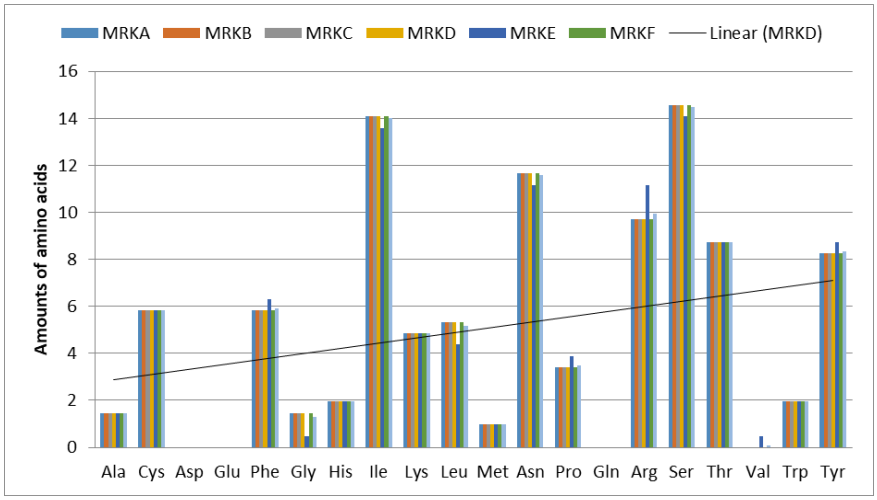


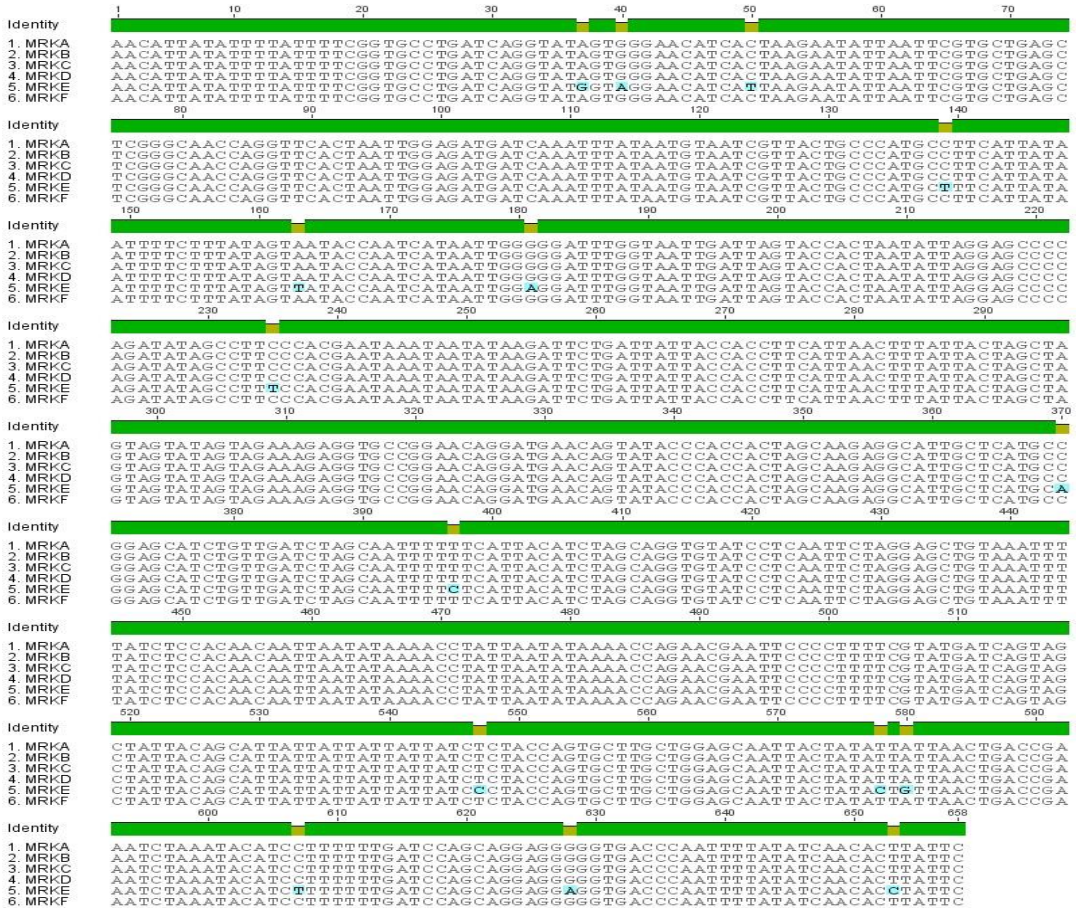
Fig 3: Comparison of the number and type of amino acids encoded by the CO1 cockroach gene from Prof. dr. Kandouw Hospital Manado, North Sulawesi, Indonesia.

Alignment

Alignment of six CO1 gene sequences of cockroaches was carried out to determine the differences in nucleotide sequences or mutations that had occurred. The alignment results with Geneous obtained 14 sites where different types of nucleotides occurred between MRKE samples and 5 samples. The alignment results showed that MRKE samples were CO1 cockroach gene samples that had undergone mutations compared to other samples which were 100% similar. The results of alignment are also supported by the results of genetic distance analysis between six CO1 cockroach gene samples. MRKE samples have 2.3% genetic distance with the other five samples. While the genetic distance between the five samples is 0 (Table 1).

Table 3: Genetic distance

	MRKA	MRKB	MRKC	MRKD	MRKE	MRKF
MRKA		100%	100%	100%	98.%	100%
MRKB	100%		100%	100%	98.%	100%
MRKC	100%	100%		100%	98.%	100%
MRKD	100%	100%	100%		98.%	100%
MRKE	98.%	98.%	98.%	98.%		98.%
MRKF	100%	100%	100%	100%	98.%	



Blast

Based on BLAST results at the NCBI site, the CO1 MRKA, MRKB, MRKD, and MRKF gene sequences have the closest similarities, with *Periplaneta americana* [KY014630.1] reported from China. In the other hand, the MRKE sequence has the closest similarity, with *Periplaneta americana* [KM576926.1] reported from the United States (Table 2). However, based on BLAST results the five CO1 gene sequence cockroaches have the closest similarities to *Periplaneta americana.*

Table 4: Similar sequences of BLAST results

No	Sample	Simililarity sequences results from BLAST NCBI	Assesion Number	Author
1	MRKA	*Periplaneta americana* cytochrome oxidase subunit I-1 gene, partial cds; mitochondrial	KY014630.1	Gao,X., Wei,D. and Xiao,L. Submitted (21-OCT-2016) College of Plant Protection, Southwest University, 2 TianSheng road Beibei District, Chongqing, Chongqing 400715, China
2	MRKB	same as the above	same as the above	same as the above
3	MRKC	same as the above	same as the above	same as the above

4	MRKD	same as the above	same as the above	same as the above
5	MRKE	*Periplaneta americana* voucher JX262 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	KM576926.1	von Beeren,C., Stoeckle,M.Y., Xia,J., Burke,G. and Kronauer,D.J. Submitted (16-SEP-2014) Laboratory of Insect Social Evolution, The Rockefeller University, 1230 York Avenue, New York City, NY 10065, USA
6	MRKF	Periplaneta americana cytochrome oxidase subunit I-1 gene, partial cds; mitochondrial	KY014630.1	Gao, X., Wei, D. and Xiao, L. Submitted (21-OCT-2016) College of Plant Protection, Southwest University, 2 TianSheng road Beibei District, Chongqing, Chongqing 400715, China

Phylogeny Construction

Based on the phylogeny tree formed from the six CO1 gene sequences, two monophyletic groups were obtained. The phylogeny tree with the neighbor-joining method formed showed an evolutionary relationship between CO1 cockroach gene sequences. MRKE samples showed differences with the other five samples because they were not in a monophyletic group. The MRKA sample forms one node with the MRKD sample while the MRKB sample forms one node with the MRKF sample. The position in one node indicates a close phylogeny relationship. Although it is in a monophyletic group with MRKA, MRKD, MRKB, and MRKF, the MRKC sample has shown a difference. This is evidenced by the MRKC position in the phylogeny tree which forms its own branching or not in one node with another sample (Figure 4). Thus there has been a variation of the cockroach CO1 gene from Prof. Dr. Kandou Hospital, Manado, North Sulawesi, Indonesia.

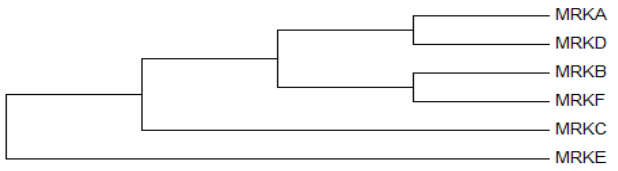


Fig 4: The cockroach phylogeny tree from Prof. Dr. Kandow Hospital, based on the CO1 gene. The phylogeny tree was built using MEGA 6.0 with the Neighbor-Joining model, Bootstrap 1000 x.

Th Beccaloni and Eggleton 2013e phylogeny tree was also built using 19 similar sequences of BLAST results on the NCBI website. Two monophyletic groups are formed. The six cockroach samples from Prof. Dr. Kandow hospital were in a monophyletic group. The phylogeny tree formed confirms that cockroaches from RSUP Dr. Kandow were included in the *Periplaneta americana* species.

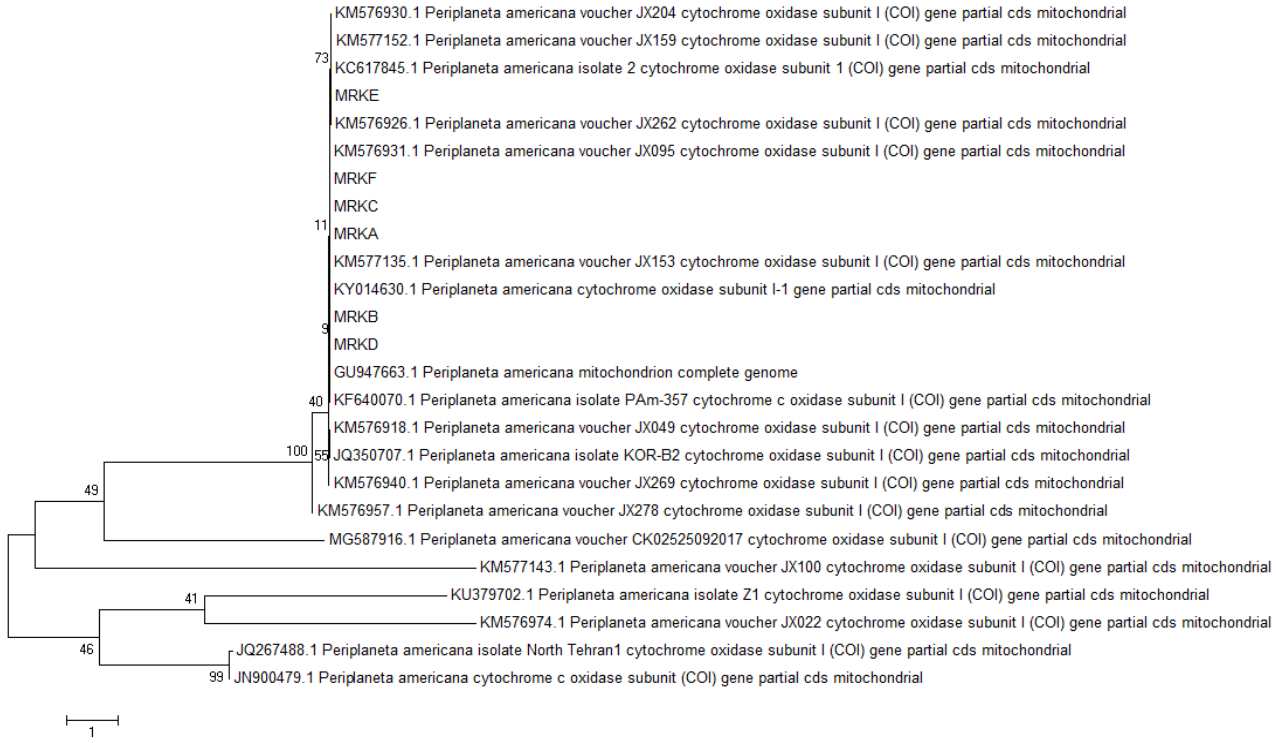


Fig 5

Conclusion

Based on the CO1 gene, cockroaches from RSUP Prof. Dr. Kandow Manado have similarities or closest evolutionary relationships to *Periplaneta americana*. MRKE samples have shown the genetic variation of the CO1 gene.

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