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The position of the species of cat lice (*ctenocephalides felix*) based on molecular barcoding of the sub unit 1 (CO1) cytochrome oxidase gene

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Abstract
The position of species of cat flea is still a lot of debate. Cat flea are one of the insect species that succeeded in converging evolution, among others, caused by human-assisted migration. Identification using morphological data is not enough to determine the position of species of cat flea. Research has been conducted to find out the position of species of cat flea from the city of Manado. The stages of the research are cat flea sampling, DNA extraction and purification, amplification and visualization of the CO1 gene, sequencing and sequence analysis. The results of this research showed based on sub unit 1 (CO1) cytochrome oxidase gene, cat flea isolated from cats in Manado city have a similarity of 99% with the CO1 gene sequence Ctenochepalides felis felis [KP687811.11]. However, the phylogeny tree formed shows younger Ctenochepalides felis from Manado based on evolutionary time. The molecular barcoding profile of the C. felis CO1 gene cannot be constructed on the BOLD system (www.boldsystems.org).

Keywords: cat flea, cytochrome oxidase gene, barcode, Manado

Introduction
Cat flea is also members of the genus Ctenocephalides until now considered the most successful ectoparasites on earth. *Ctenocephalides* sp. (Siphonaptera: Pulicidae) (Bouché), is the most common species of flea found in cats and dogs throughout the world. The widespread parasitization of these insects in mammals is closely related to humans. As pets, dogs and cats have a significant potential for disease transmission caused by pathogenic microbes (Lawrence, et. al., 2014; Lawrence et.al. 2015; Bahrami et. al. 2012) [9, 10, 3]. The spread of fleas from the genus Ctenocephalides is much assisted by humans who move between continents or countries by carrying cats or dogs. On the other hand, it causes high morphological and genetic variations in the genus Ctenocephalides (Lawrence, et. al., 2014; (https://cameronwebb.wordpress.com/tag/ctenocephalides-felis/) [9].

The University of Sydney, Department of Medical Entomology, has collected and researched Ctenocephalides from around the world. Morphological, morphometric and molecular identification studies were carried out on 1000 samples from 50 countries. The focus of their research is the characteristics of cat fleas that migrate in various continents through cats as pets. Research conducted over seven years found many morphological and genotypic variations, especially mitochondrial DNA in Cat flea. Similar research was also conducted by Lawrence et. al. 2015, against cat fleas from Australia, Fiji, and Thailand. However, genotype has never been analyzed for cat fleas from Sulawesi. The results of the study with molecular barcoding techniques, using mitochondrial DNA of CO1 and COII genes, obtained that each cat louse from three countries formed different phylogeny tree clusters. In both morphological and molecular approaches, variations of Ctenocephalides felis were found. The morphological similarity between C. felis and C. canis is a challenge for entomologists. There is still much debate about the morphological characteristics and position of the species Ctenocephalides sp. (Lawrence, et. al., 2014) [9, 10].

Until now there has been no research report about the position of species of cat flea from Manado. At present Manado has developed into an important tourist area in Indonesia and an intercontinental transit area. Data from the North Sulawesi provincial animal quarantine center in 2017 shows that many tourists carry pets, especially cats. On the other hand, data from the veterinary services of the city of Manado, an increase in the cat population of 17.5% every year since 2017.

Materials and methods
Sample
Cat flea samples were obtained from four locations in the city of Manado. Cat fleas are obtained from Ranotana, Karombasan (traditional markets), Malalayang and Pal two. Cat flea is isolated directly from the cat's body. Cat fleas obtained are preserved in sample bottles containing 95% alcohol. After 24 hours, Cat flea are transferred to a new sample bottle containing 95% alcohol. Each location was taken 10 cat fleas without distinguishing the sexes.

Extraction and Purification Genomic DNA of C. felis
A 30 mg of adults Ctenocephalides felis from Manado then transfer it to a 1.5 ml microcentrifuge tube. Add 200 µl of GT Buffer to the tube and homogenize the sample tissue by

grinding. Add 20 µl of Proteinase K to the sample mixture then shake vigorously and incubate at 60ºC for 30 minutes. During incubation, invert the tube every 5 minutes. Add 200 µl of GBT Buffer then shake vigorously for 5 seconds. Incubate at 60ºC for at least 20 minutes to ensure the lysate is clear. During incubation, invert the tube every 5 minutes. If insoluble material is present following incubation, centrifuge for 2 minutes at 14-16,000 x g then transfer the supernatant to a new 1.5 ml microcentrifuge tube. At this time, preheat the required Elution Buffer (200 µl per sample) to 60ºC (for Step 4 DNA Elution). Add 200 µl of absolute ethanol to the lysate then immediately shake vigorously for 10 seconds. Place a GS Column in a 2 ml Collection Tube. Transfer the mixture (including any precipitate) to the GS Column then centrifuge at 14-16,000 x g for 2 minutes. • Discard the 2 ml Collection Tube then transfer the GS Column to a new 2 ml Collection Tube. Add 400 µl of W1 Buffer to the GS Column then centrifuge at 14-16,000 x g for 30 seconds. Discard the flow-through then place the GS Column back in the 2 ml Collection Tube. Add 600 µl of Wash Buffer (make sure ethanol was added) to the GS Column. Centrifuge at 14-16,000 x g for 30 seconds. Discard the flow-through then place the GS Column back in the 2 ml Collection Tube. Centrifuge for 3 minutes at 14-16,000 x g to dry the column matrix. Transfer the dried GS Column to a clean 1.5 ml microcentrifuge tube. Add 100 µl of pre-heated Elution Buffer or TE to the center of the column matrix. Let stand for at least 5 minutes to ensure the Elution Buffer or TE is completely absorbed. Centrifuge at 14-16,000 x g for 30 seconds to elute the purified DNA.

Amplification and visualization of the CO1 gene amplicon.

Amplification of the COI gene was carried out by polymerase chain reaction using real time PCR - rotor gene Q Series. A total of 2 l of DNA Ctenocephalides felis was used as PCR template. Other PCR components that have been used are shown in table 1. Samples are included in the Q gene series rotor cube and are run with the Q gene series software 2.0.3 rotor. The PCR conditions applied were the stages of initiation of denaturation 940C for 3 minutes, the denaturation stage of 940C for 30 seconds, the annealing stage 520C for 50 seconds, the extension stage 720C for 1 minute and the final extension 720C for 5 minutes. A number of cycles 35 times. Visualize the amplicon CO1 gene using 0.8% agarose electrophoresis.

Table 1: PCR Components

Komponen	Jumlah
Top tag master mix Qiagen	7,5 µl
Primer forward	2 µl
Primer reverse	2 µl
ddH_2O	6,5 µl
DNA *C. felis* from Manado city	2 µl
Total	25 µl

Sequencing

Amplicon with a stable band (no smears), with a length ranging from 550 bp - 800 bp, followed by sequencing. Sequencing is done at First Base Laboratory, Singapore. Sequencing using the PRISM 3730xl Genetic Analyzer ABI Develop by Applied Biosystems, USA.

Analysis of the CO1 gene sequence

Sequencing data obtained were analyzed by Geneious 10.1.1 software (Kearse *et al*., 2012) [7] and the Molecular Evolutionary Genetics Analysis (MEGA) program (Tamura et. al., 2013) [15]. The sequence obtained is then carried out homology analysis using the basic local alignment search tools (BLAST) (Astchul *et al*., 1990; Donkor et.al. 2014). BLAST is conducted online at the national center of biotechnology information (NCBI) website. The reconstruction of the phylogeny tree was carried out by the neighbor-joining model. The calculation of the genetic distance matrix with the Kimura-2 parameter model is implemented in the pairwise distance calculation with Bootstrap 1000 replications in the MEGA software version 7.0. The CO1 sequence of Ctenocephalides felis from Manado was compared to 100 BLAST sequences on the NCBI website.

Results and discussion

The total DNA of Ctenocephalides felis was successfully extracted using Geneiad's Genomic DNA mini kit (Tissue). The total DNA purity was A260 / 280 which was 1.76 while the total DNA concentration was 37.5 µg / µl. Amplicon obtained by the PCR method visualized at 530bp - 820 bp. This result is in accordance with the estimated length of the CO1 gene (Herbert and Gregory, 2005; Hebert et.al. 2003) [5, 6].

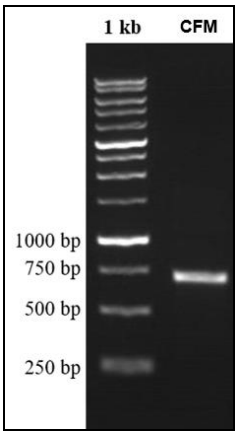
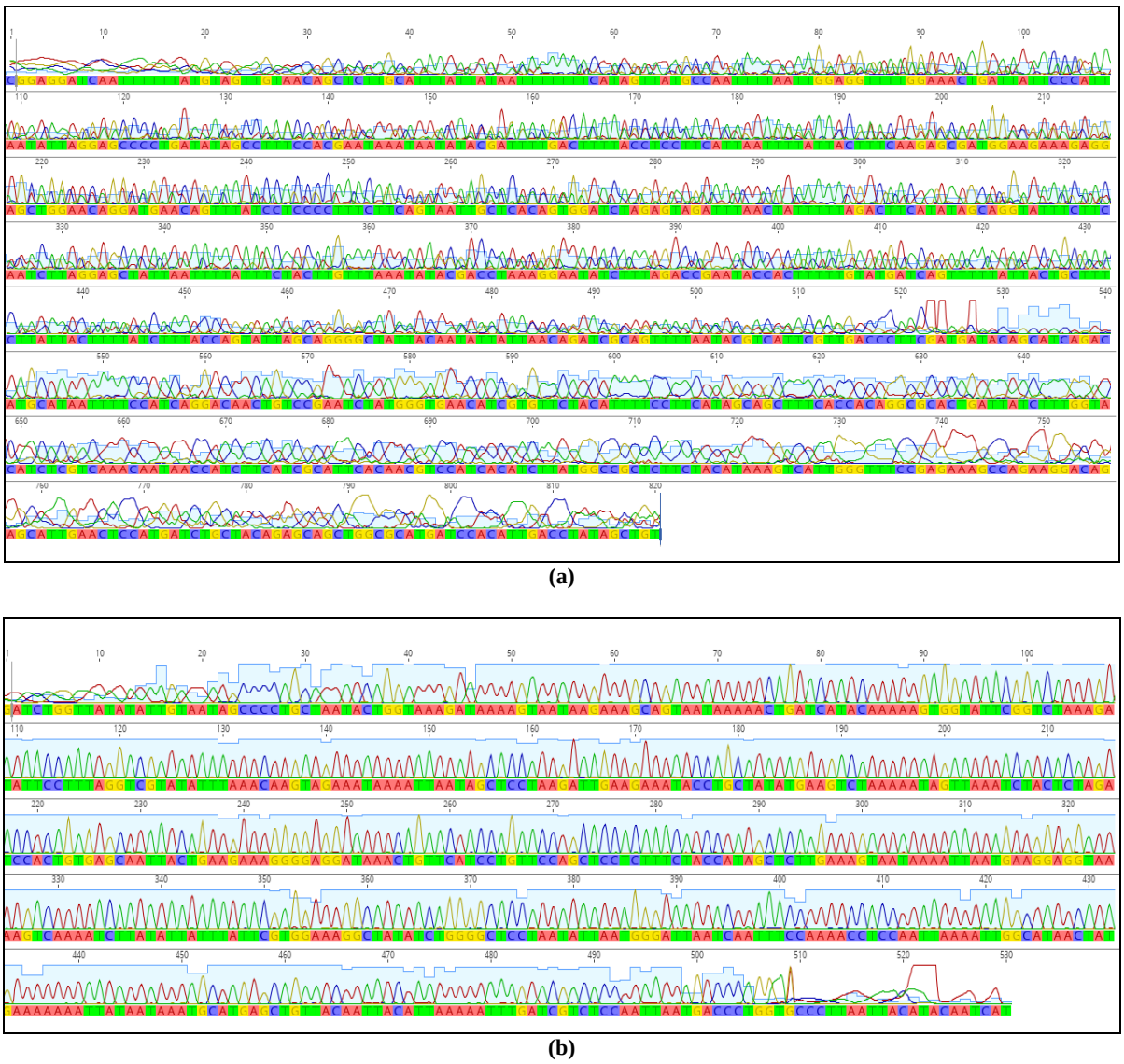


Fig 1: Visualization of the amplicon of the C. felis CO1 gene from Manado (CFM: Ctenocephalides felis Manado).

The application of the CO1 gene as a complement to the identification of insect morphology found in North Sulawesi has been successfully carried out on *Apis dorsata* Binghami (Sulawesi endemic honey bees) (Mokosuli, 2013) [11], house flies (Rotty et.al. 2018, Kanan and Tulung, 2017), *Anopheles* sp (Manuahe et. Al. 2016) [12], *Aedes* sp (Timah and Mokosuli, 2017) [16] and local fruit flies (Sumampouw et.al. 2017) [14]. Primary CO1 gene proposed by Folmer et.al. 1994 sensitive in identifying the insect. The success of total DNA extraction and purification largely determines the amplification of the CO1 gene. A common problem in extracting insect DNA is the number of contaminants from eksoskleton (Mokosuli, 2013; Manuahe, 2016) [11, 12]. Because of the small size of adults *Ctenocephalides felis*, the whole body is used as a tissue source for total DNA extraction. Modification of proteinase-K immersion and senstrifuse technique has been proven, can increase the concentration and purity of total insect DNA (Manuahe et.al. 2017) [12].

The results of the sequencing of the CO1 Ctenocephalides felis gene by sequencing method obtained a good chromatogram (Figure 3 and Figure 4). This is indicated by a chromatogram band that only slightly coincides with each other. The red ribbon shows the Adenine nucleotide, the yellow ribbon shows the guanine nucleotide, the blue ribbon shows the cytosine nucleotides and the green ribbon shows the thymine nucleotides (Figure 2).



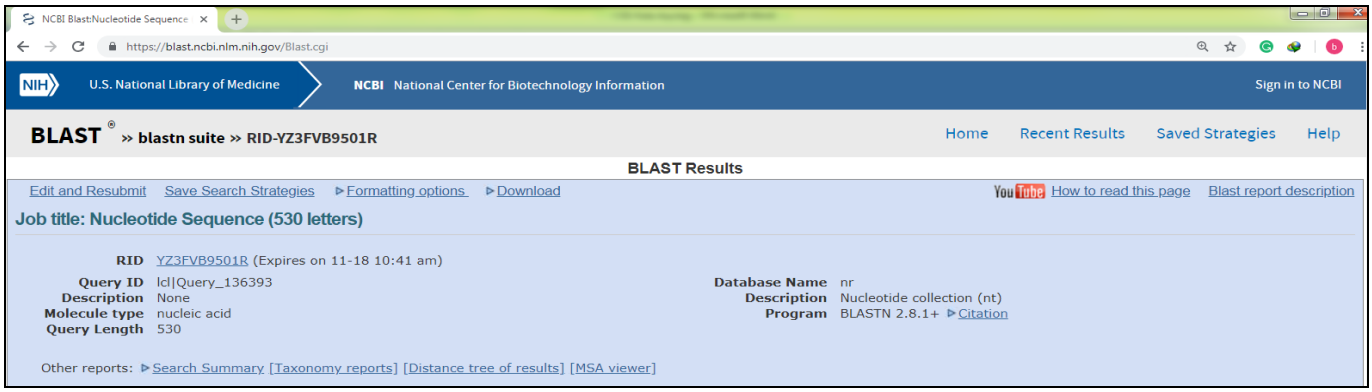
(a)

(b)

Fig 2: Chromatogram results of CO1 gene ctenocephalides felis sequencing, a. Forward and b. Reverse.

Blast

Alignment of the CO1 Ctenocephalides felis gene sequence to obtain the same sequences recorded in the NCBI gene bank has been done using BLAST (www.ncbi.gov). The BLAST results obtained 100 similar sequences with a ratio of more than 200 nucleotides (Figure 20).



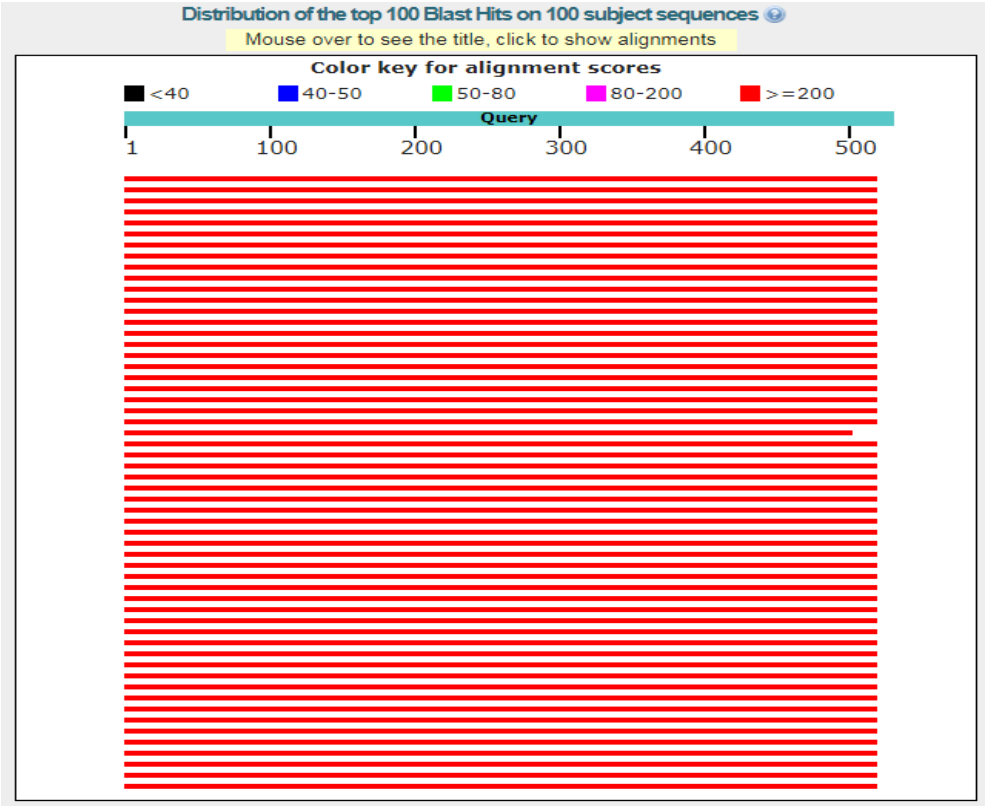


Fig 3: Results of C. felis CO1 gene alignment using NCBI BLASTn

Based on the results of BLAST, it was verified that cat fleas originating from the city of Manado were Ctenocephalides felis with a similarity of 99%. This is reinforced by 99 sequences of BLAST results showing species of Ctenocephalides felis. Only one BLAST result refers to Ctenocephalides canis (Figure 3). BLAST's taxonomy report obtained 186 Ctenocephalides, 90 Ctenocephalides felis, 90 Ctenocephalides felis felis, 5 Ctenocephalides felis strongylus, and 1 Ctenocephalides canis. This report taxonomy shows that C. felis still has an evolutionary relationship close to C. felis; the similarity of CO1 nucleotides more than 200 nucleotides. Ctenocephalides felis from Manado has the closest similarity to C. felis [KY417923.1], which comes from..... (Figure 3).

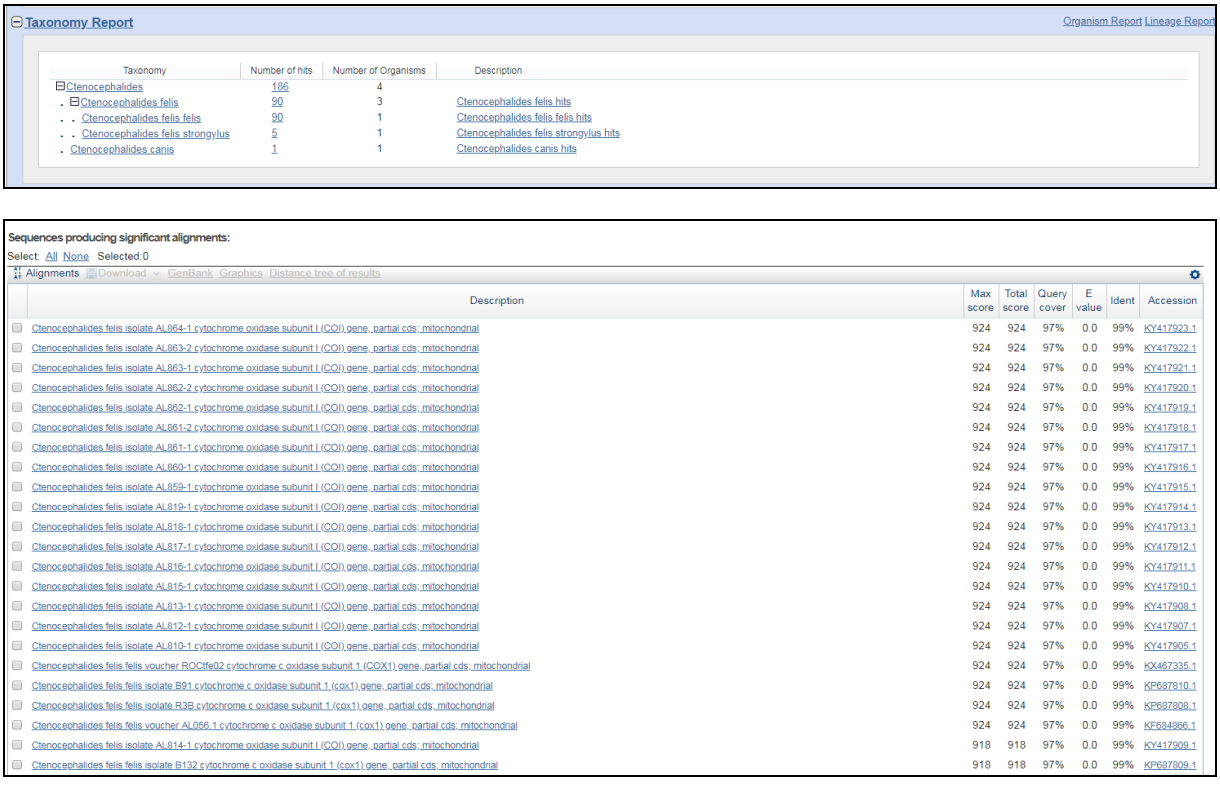


Fig 4: The BLAST CO1 C. felis gene sequence results from the city of Manado

Determination of molecular barcodes is carried out on the BOLD site (Figure 5). However, the C. felis molecular barcode is not successful because there is no C. felis data in the gene bank BOLD system. Thus, it is necessary to report the sequence of the C. felis CO1 gene in order to have a gene database on the BOLD system.

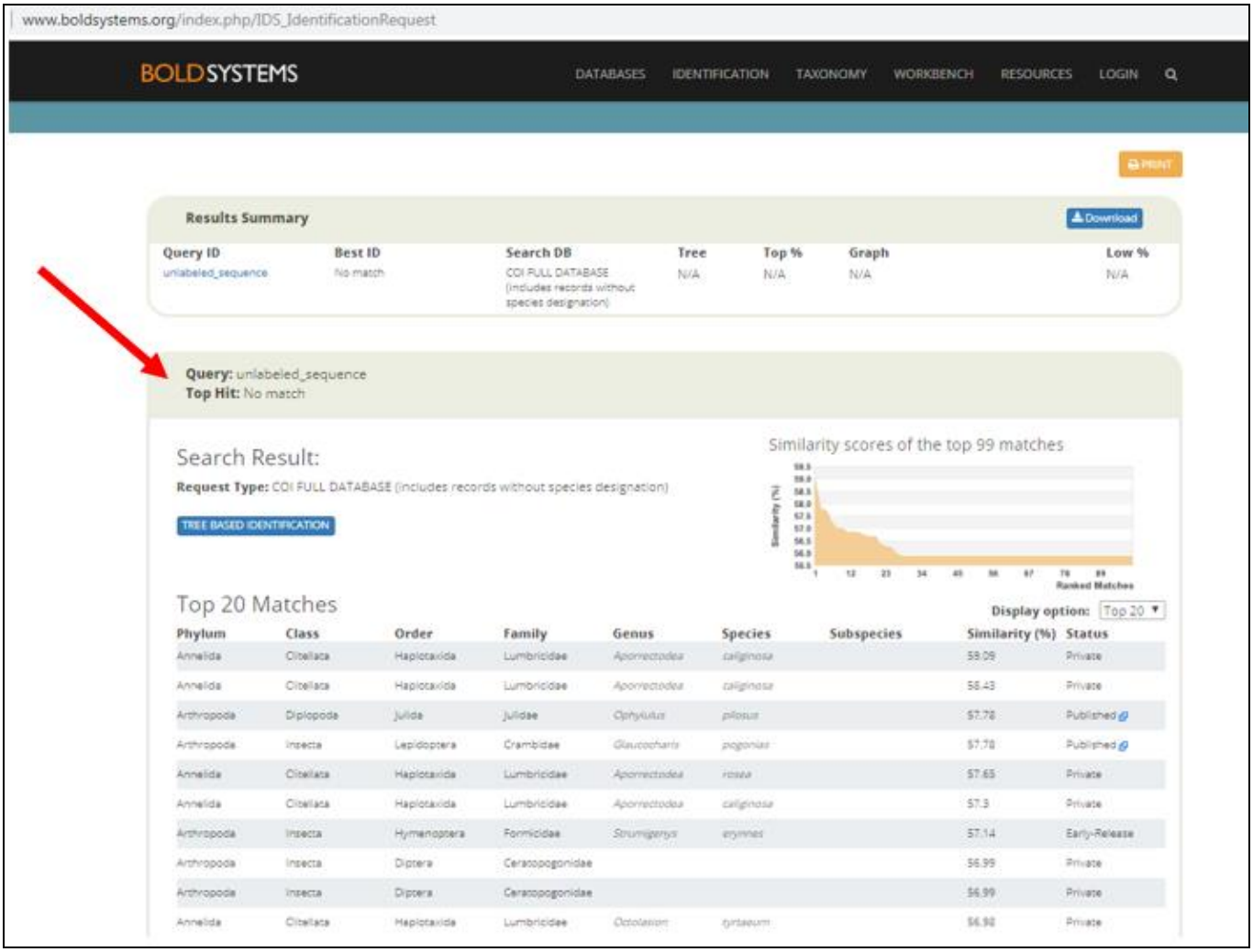


Fig 5: Barcoding results on the C. felis CO1 gene from Manado on the website www.boldsystems.org

Construction of phylogeny trees.

Construction of the phylogeny tree, carried out online at the NCBI website (www.ncbi.gov). The construction of the phylogeny places the position of Ctenocephalides felis from the city of Manado as having the closest evolutionary relationship with Ctenochepalides felis felis [KP687811.11] (Figure 5). The phylogeny tree constructed using 100 CO1 gene sequences from BLAST formed two monophyletic groups. The position of Ctenocephalides felis from Manado is in the first monophyletic group. The first monophyletic group still forms 2 clades of subgroups. Based on the position of Ctenocephalides felis from Manado on the phylogeny tree, although it forms one node with [KP687811.11] it has many differences. The position of Ctenocephalides felis from Manado is further away from ancestors or younger based on evolutionary time.

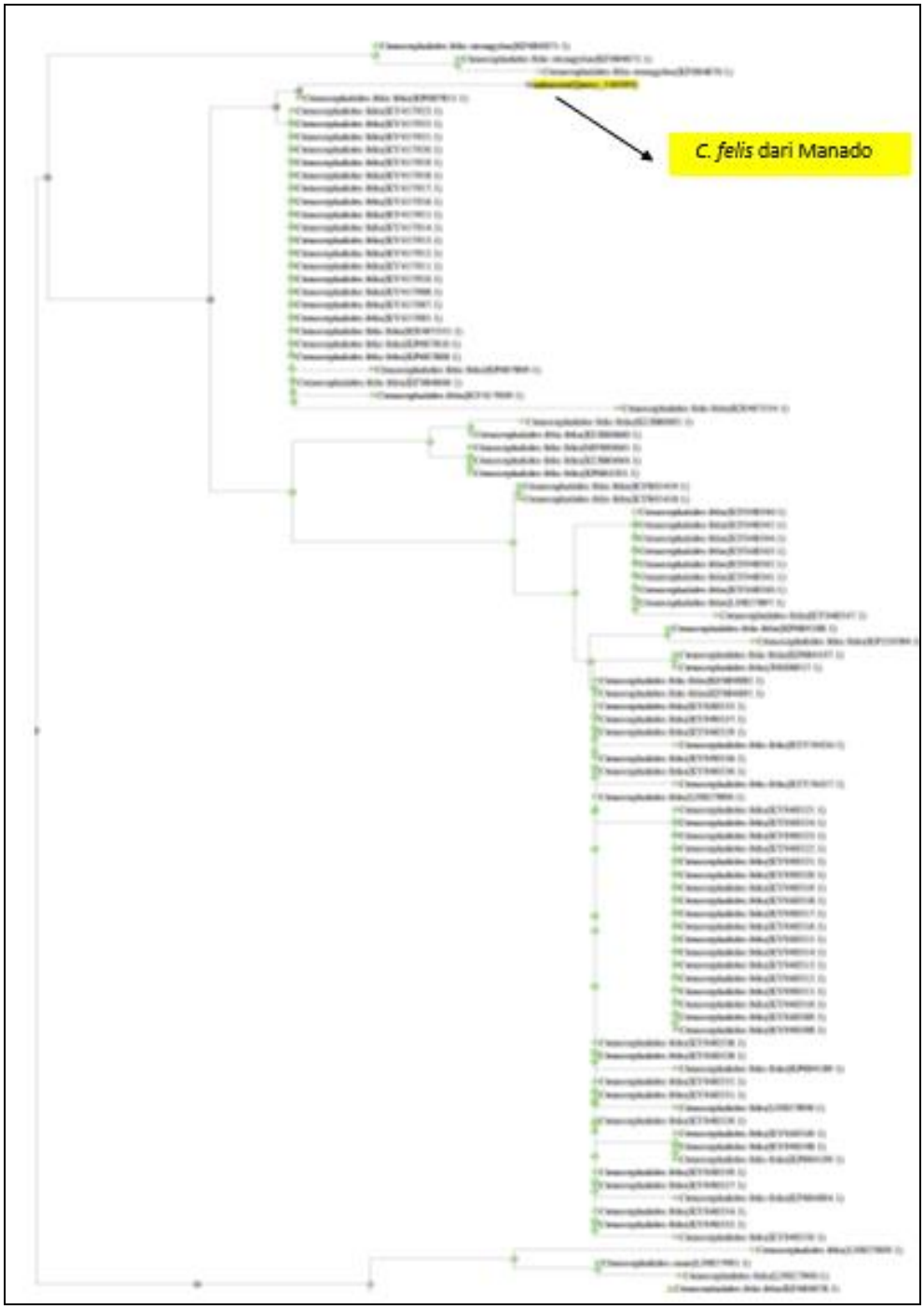


Fig 6: The phylogeny tree Ctenocephalides felis from Manado was built with a 1000x Neighbor Joining bootstrap model.

Conclusion

Based on subunit 1 (CO1) cytochrome oxidase gene, cat flea isolated from cats in Manado city have a similarity of 99% with the CO1 gene sequence Ctenocephalides felis felis [KP687811.11]. However, the phylogeny tree formed shows younger Ctenocephalides felis from Manado based on evolutionary time. The molecular barcoding profile of the C. felis CO1 gene cannot be constructed on the BOLD system (www.boldsystems.org).

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