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DNA Barcoding of local pigs in minahasa, north sulawesi

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Abstract
DNA barcoding and Molecular phylogeny reconstruction of local pigs from Minahasa North Sulawesi, Indonesia was investigated using partial sequences of Cytochrome c oxidase subunit I gene (CO1). Two location according the place of traditional pigs farm used this study that is Minahasa Utara and Minahasa was analysis the partial CO1 gene. Phylogeny trees was constructed online in NCBI site. From the results of this studied, based on partial CO1 gene, local Pigs of Minahasa cannot be sure of the position of species, because it does not have a sequence similar to the gene bank. The North Minahasa local pigs showed the similarity of the nearest CO1 sequence to the Pig DNA sequence from clone WTSI_1061-78D9. Similarly, the phylogeny construction shows the closest kinship based on the CO1 gene with Pig DNA sequence from clone WTSI_1061-78D9.

Keywords: local pigs, minahasa, CO1 gene, DNA barcoding

Introduction
The pigs are farmed by the Minahasa community, consisting of local pigs and hybrid pigs. Large-scale breeders, preferring hybrid pigs because of their large body size to be able to provide meat in larger quantities, than smaller Minahasa local pigs. Feed for hybrid pigs, generally in the form of synthetic pellets. However, feed for local pigs that are farmed on a small scale using the remaining agricultural produce, among others: tubers, corn, rice bran (konga) and others - generally derived from nature.

The Minahasa sub-ethnic group has been raising pigs for a long time. Pigs have been raised in Minahasa since the 18th century (Smith, 1958) [13]. Preliminary research has been done, found morphological differences between local pigs in Minahasa region. Morphological variations include the shape of the mouth, the shape of the ear, the shape of the foot and the shape of the nose. Morphological variations occur, among others, due to the culture of Minahasan people doing cross-breeding between local pigs. In rural communities, pig farms do not use cages. Thus, it is suspected to have cross-breeding between local pigs and wild pigs. However, there has not been much research on the species and genetic diversity of local Minahasa pigs. Other local pig studies have been conducted in Indonesia including local Batak pigs, local Krawang pigs, local Nias pigs and local Toraja Land Pigs. Local pig identification is more conventionally done. This leads to the need for experts and a considerable amount of time to establish the status of local pig species in Indonesia.

The method analysis of the position of the species of an organism that is currently the most widely performed is the analysis of mitochondrial DNA. The cytochrome oxidase sub unit 1 (CO1) is most widely used as a marker or genetic barcode (Mokosuli *et al* 2014). Mitochondrial DNA analysis (mtDNA) is one of the most widely used methods for studying the origins of domesticated livestock (Machugh and Bradley, 2001) [16]. In mammalian mitochondrial DNA only psassed through the female (maternal) without recombination with sperm (male). Mitochondrial sequences have been used to study the origins of cattle (Troy *et al*., 2001) [34], pigs (Giuffra *et al*., 2000) [6], sheep (Hiendleder *et al*., 2002) [7], horses (Villa *et al*., 2001), dogs (Savolainen *et al*., 2002) [24], donkeys (Bejapreira *et al*., 2004) and pigs (Joshi *et al*., 2001; Luikart *et al*., 2001; Mannen *et al*., 2001; Sultana *et al*., 2003; Chen *Et al*., 2005; Naderi *et al*., 2007; Royo *et al*., 2009; Zhao *et al*., 2011) [13, 14, 17, 31, 4, 18, 21]. This research has earned the status of local pig species in Minahasa and North Minahasa.

Materials and method

Samples

Sampling will be conducted in 2 regions: Minahasa, and North Minahasa (Figure 1). The local pigs who were the source of the sample were identified by experts from the Department of Biology FMIPA Manado State University. Local pig tissue samples, which have been used in this study are the thigh and tail tissue.

Fig 1: Location of local pigs Minahasa Sampling

DNA Extraction, PCR Amplification and Sequencing

Total genomic DNA was extracted from legs and tail muscle tissue samples using Genomic DNA Mini Kit (Tissue) according to the manufacturer's protocol. Ampolification of CO1 gene using PCR method was applied MyTaq HS Red Mix Bioline (Table 1 and Table 2).

Table 1: The Componets of PCR

PCR Component	Volume (µL)
2x MyTaq HS Red Mix Bioline	25
Primer Forward: LCO1490 : 5'-GGTCAACAAATCATAAAGATATTGG-3' (Folmer *et al.*, 1994)	1
Primer Reverse : HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al.*, 1994)	1
DNA Template	2
DdH_2O	21
Total	50

Table 2: The Conditions of PCR

Cycle	Time (Second)	Temperatur (°C)	Phase
35 x	60	94	Denaturasi
	30	50	Annealing
	30	72	Ekstension
	60	72	Final Ekstension

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) [33]. Phylogenetic trees were reconstructed on line on the NCBI website.

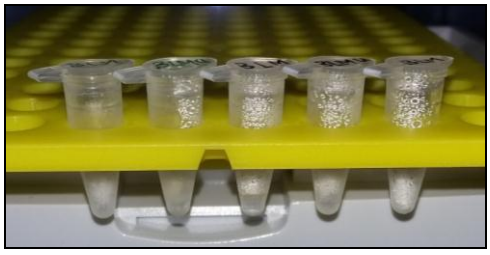
Results and discussion

1. Extraction dan Purification of Total DNA of Pigs

Legs muscle tissue and tail has been used for DNA extraction and purification. The samples were preserved with 70% ethanol. The mean sample weight was 29.6 mg (Table 2). The total DNA concentration of samples from Minahasa was 13.7 µg / ml with a purity of 1.2 whereas the total DNA concentration of samples from North Minahasa was 9.75 µg / ml with a purity of 0.6.

A

B



C

Fig 2: (a) Minahasa Local Pig, (b) Legs Muscle Tissue in 70% ethanol, (c) Total DNA of Minahasa Local Pigs.

Tabel 4: Concentration and Purity of Total DNA

S. No	Sampel	Asal Jaringan	Berat Sampel	Waktu Inkubasi (Proteinase K)	Konsentrasi DNA	Kemurnian	Jumlah DNA
1	Minahasa	Legs Muscle Tissue	29,5 mg	Incubated at 60 ^{0} C for 40 Minutes	13,7	1,2	100 µl
2	Minahasa	Legs Muscle Tissue	29,5 mg	Incubated at 60 ^{0} C for 24 Hours	20,3	1,6	100 µl
3	Minahasa Utara	Tail Muscle Tissue	29,7 mg	Incubated at 60 ^{0} C for 40 Minutes	9,75	0,6	100 µl
4	Minahasa Utara	Tail Muscle Tissue	29,7 mg	Incubated at 60 ^{0} C for 24 Hours	21,50	1,55	100

2. Amplifikasi dan Visualisasi Amplikon Gen COI

Amplification of CO1 gene reads respectively at lenght 583 bp and 981 bp. Accordingly bands that formed showed the high concentration of amplicons partial CO1 gene in all sample (Figure 3).

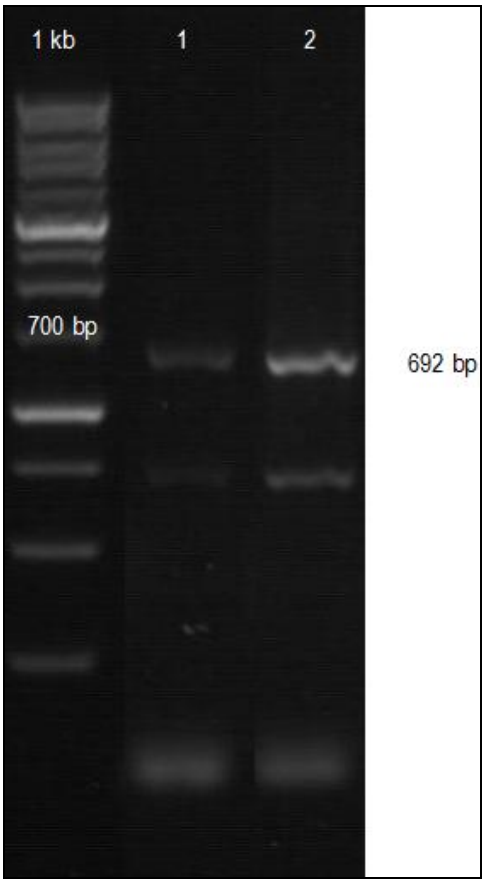
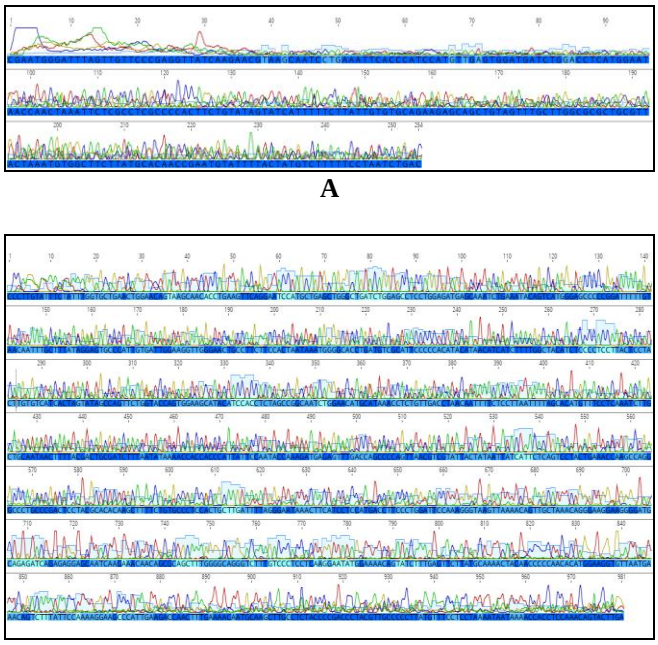


Fig 3: CO1 gene amplification of Local Pigs Minahasa Sample on 1,5% agarose gel.

Lanes 1: kb as a marker, 1 = Local Pigs from Minahasa, 2 = local pigs from Minahasa Utara

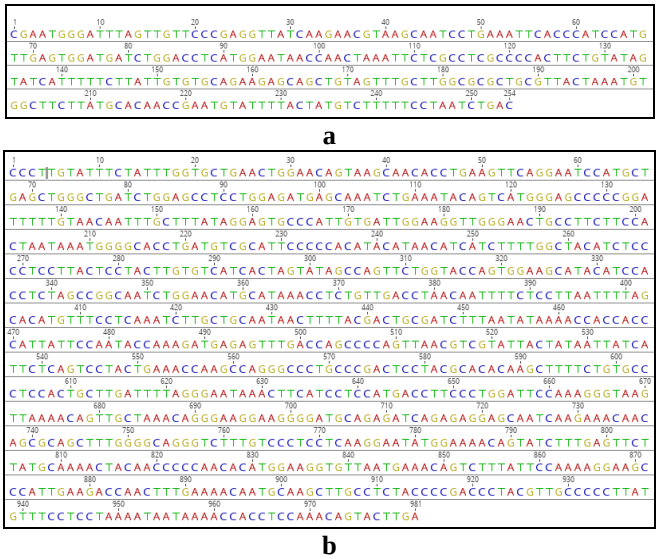
Based of the chromatogram were resulted of the sequencing, showed all sequens of partial CO1 gene are good. The formed chromatogram showed, the tops of the bands are perfectly separated between the nitrogen base types (Figure 2). The sequenced file sequencing data from First Base Singapore is analyzed using the Geneious 10.1.3 (Drummond *et al*. 2012), to obtain the CO1 gene sequence of the Minahasa and North Minahasa Local Pigs. The Minahasa local pig sequence in the FASTA format is further analyzed alignment with the BLAST method on the NCBI website. The sample sequences of local Minahasa and North Minahasa North pigs have a length of 583 bp and 981 bp respectively (Figure 9).



A

B

Fig 4: Chromatogram of CO1 gene sequencing product of Minahasa and North Minahasa Local Pigs



a

b

Fig 5: Sequences of CO1 gene Local Pigs Minahasa (a) and North Minahasa (b).

Based on the analysis of CO1 gene sequence, local Minahasa pig has obtained GC ratio: 43.2%, while local Minahasa North pig is 42.2%. The highest frequency of nitrogen bases in the local Minahasa pig's CO1 sequence is Timin (30.7%), whereas in North Minahasa local pigs are Adenine (29.8%)

(Table 5). The results of the alignment analysis, the local Minahasa pig CO1 gene on the NCBI website, found no similar sequence results.

Table 5: Characteristics of CO1 Gene of Local Pigs Minahasa and North Minahasa.

Characteristics	Minahasa		North Minahasa	
	Freq.	%	Freq.	%
Nitrogen Base Composisition :				
Adenine	62	24,4	283	28,8
Cytosin	56	22,0	253	25,8
Guanin	51	20,1	181	18,5
Timin	85	33,5	264	26,9
Guanin Cytosin	107	42,1	434	44,2
Lenght Sequences (base pairs)	254		981	

Discussion

Mitochondria are cell organelles, the site of cell respiration to produce ATP as cellular energy. Mitochondria are found in muscle tissue (Nelson and Cox, 2005; Mokosuli, 2013) [19]. Therefore, muscle tissue of the legs and tail muscles, used for total DNA extraction and purification. Mitochondrial DNA is extracted using Geneaid Mini KIT (Tissue) Genomic DNA following a modified KIT protocol. The DNA concentration according to the KIT protocol is more than 10 µg and the purity is in the range 1.8 - 2.0. The results of this study showed that samples of local Minahasa pig tail muscle tissue, incubated with Proteinase K for 40 minutes, yielded a concentration of 13.7 µg and purity of 1.2, while North Minahasa Local pig's tissue samples incubated with Proteinase K for 24 Hour, yielding a concentration of 9.75 µg and a purity of 0.6. The results showed that long-period immersion of K-proteinase, had an effect on total purity and DNA purity. The duration of K-protein immersion also affects the extraction and purification of Aedes sp DNA (Manuahe *et al* 2015); Apis dorsata Binghami (Mokosuli, 2013), Tarsius sp. (Kamagi et.al. 2013).

The total DNA obtained, from the extraction and purification of DNA, was amplified using 2x My Taq HS Red Mix and COI primer by Folmer et. Al. 1994. The PCR process modification was performed on the PCR condition ie the total time of all PCR stages was 155 min, unlike the Kit protocol of 145 min. Amplification of COI gene, qualitatively shows good amplicon yield. This is evidenced by the emergence of stable bands on PCR visualization results with electrophoresis. Thus the CO1 primer used, is quite effective on a sample of local Minahasa pigs.

The results of the alignment analysis with the BLAST method, for the Minahasa local pig sequence do not show any resemblance to any sequence in the Gene Bank NCBI. According to these results, it can be presumed that the Minahasa local pig sequence is a new species that has not been previously reported. The BLAST results for the Minahasa local pig sequence can be seen in Figure 6

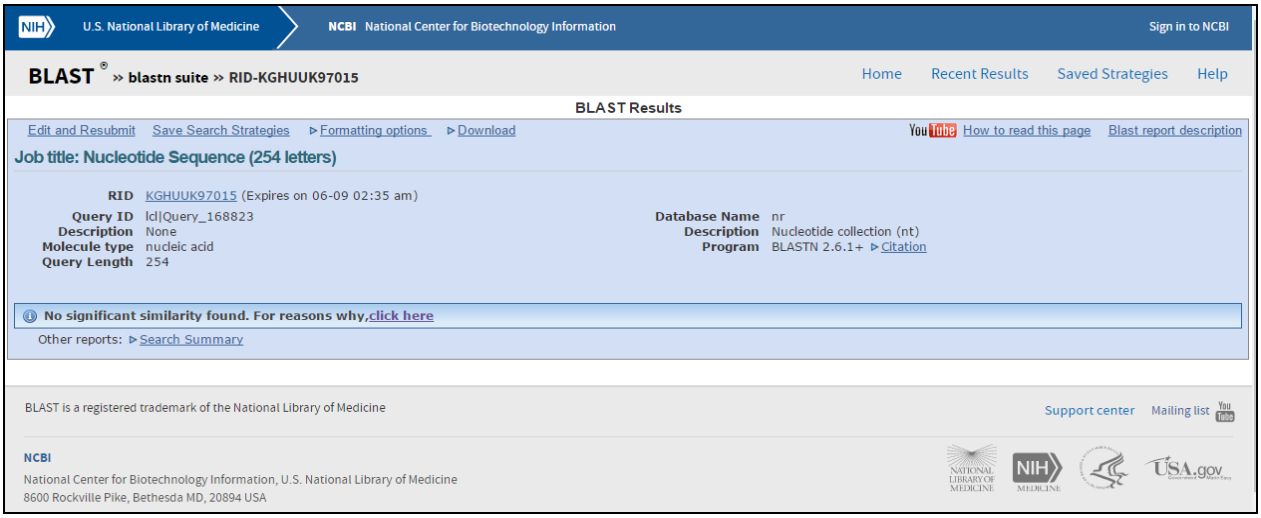


Fig 6: Results of BLAST the CO1 gene sequence of Local Minahasa Pigs

In contrast to the results of BLAST sequences of the local Minahasa pigs CO1 gene, the North Minahasa local pig gene sequence, shows, similarity 96% with Pig DNA sequence (WTSI_1061-78D9). The reconstruction of the phylogeny tree using the Neighbor Joining (1000 x bootstrap) method, has been done online at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Overall there are 17 sequences with the closest resemblance of the BLAST result in NCBI Gene Bank, used to form the phylogeny tree (Figure 7).

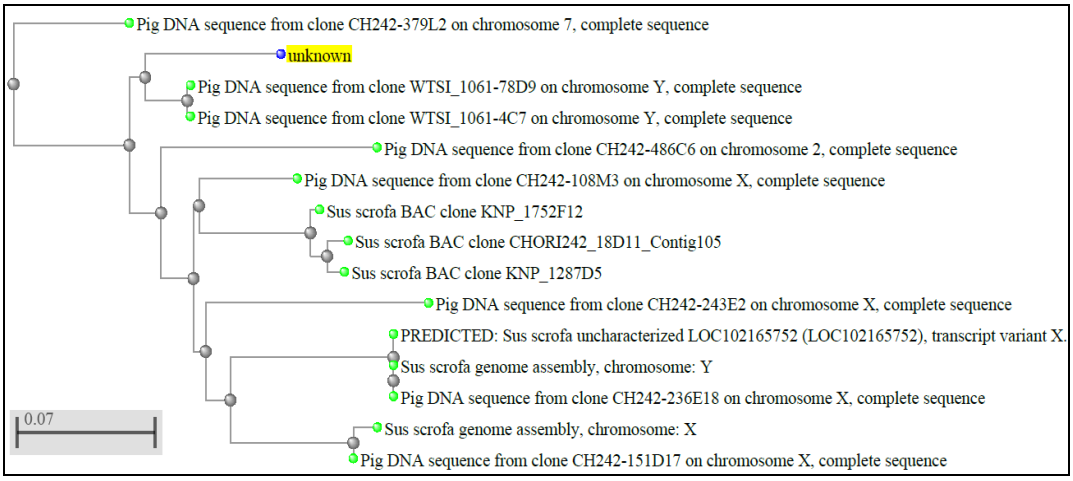


Fig 7: Rekonstruksi Filogeni Babi lokal Minahasa (Query_103899) pada situs NCBI

Conlusion

From the results of this study can be concluded, that local Minahasa pigs can not be sure of the position of species, because it does not have a sequence similar to the gene bank. The North Minahasa local pig shows the similarity of the nearest CO1 sequence to the Pig DNA sequence from clone WTSI_1061-78D9. Similarly, the phylogeny construction shows the closest kinship based on the CO1 gene with Pig DNA sequence from clone WTSI_1061-78D9.

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