CONFERENCE PROCEEDING

Phylogenetic Analysis to Goat's Milk using MEGA X: Reflection from Quran

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Abstract

Goat's milk benefits humans by providing nutrients like protein, calcium, fats, carbohydrates, vitamins, and minerals. In the holy Quran, Goat's milk is mentioned 5 times indicating its importance to humans and nature. Due to the study of mislabelling of dairy products globally, the authenticity and traceability of commercialised milk are often questioned among the public. In the case of goat milk products, clear and accurate labelling is recommended with respect to the geographical origin of the products and the substances used in the products. This is to make sure that the correct decisions can be made by consumers. To investigate the phylogenetic relationship in this analysis, DNA sequence data and mitochondrial nucleotide sequences of raw (self-milking) and commercialised goat milk (*Capra aegagrus hircus*) from each state of Malaysia were used. For the reconstruction of the phylogenetic tree, Neighbour-Joining (NJ), Maximum Parsimony (MP) and Molecular Evolution were used. Following the manufacturer's (Promega, USA) protocol description, genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit. By using Clustal X, multiple sequence alignment was performed. In addition, qualitative methodology of content analysis is also carried out. The congruent findings of the trees for *Capra aegagrus hircus* indicate that the commercialised samples of goat's milk are considered to be authentic based on the phylogenetic tree of our study. To our knowledge, there is still no recent study on the validity of goat milk in Malaysia, and this understanding will play an important role in educating customers about the authenticity of goods. Interestingly, Islamic perspectives are also studied for the benefits of society especially Muslims, hence increasing the development of integration of Naqli and Aqli knowledge.

Keywords: *GENIUS INAQ, Goat's Milk, Phylogenetic Analysis, MEGA X*

INTRODUCTION

As its substances complete the food pyramid, goat's milk and other dairy substances are essential for one's daily food consumption. In particular, goat milk has a high nutritional value for health maintenance, such as calcium, phosphorus, chlorine and various vitamins (Hipni et. al.,2020). While cow's milk is more widely sold and consumed by people, because of its comparable nutrient content, goat's milk may be a recommended substitute for cow's milk for those with an allergy. However for some diseases such as diabetes mellitus, because of its lower sugar content, goat's milk is preferred to cow's milk (Verruck et. al., 2019). In comparison, goat milk often has a lower content of fat and makes it ideal for those with high blood pressure (Kalyankar

et. al., 2016). In addition various goat breeds have been shown to have different concentrations of milk fatty acids and should be taken into consideration by customers for particular health benefits (Hakim et. al., 2019). Goat milk is also preferred to be consumed as a whole by humans.

Goat's milk industry has expanded globally around 70% somewhere in the range of 1991 and 2011 (FAOSTAT, 2013) particularly in the Mediterranean region and the Center East. Goat's milk plays a significant function in one's way of life and climate, which appeared in nations like France, Italy, Spain, and Greece (García etl al., 2014). In Southeast Asian nations, commercial dairy goat's farm production is effectively expanding because of the interest for goat items, for example, milk and meat (Liang & Devendra, 2014). Despite the fact that there is no official recorded information of goat's milk creation and utilization in Malaysia, however the interest for goat's milk is emphatically expanding since the public begin to recognize the advantages of the milk on wellbeing (Shahudin et. al., 2018). Goat's milk is exceptionally bought and devoured by local Muslim buyers according to descriptive statistics (Kamarubahrin, 2019). This is because of the accompanying Prophet Muhammad (PBUH) Sunnah and as a result of its nutritious and sound substance. Overall, goat's milk industry could be exploited for industrialization of a nation, and indeed contributes a great deal in various nations' economy particularly in the Mediterranean, Center East, Eastern Europe, and South American nations (Ribeiro & Ribeiro, 2010; Bhattarai, 2012). Begun in 1950, dairy goat farming in Malaysia utilized imported breeds, for example, Saanen, Anglo Nubian, British Alpine, and Jamnapari (Shahudin et. al., 2018).

The purity of commercialised goat's milk has also been challenged, as it is the most critical part of the defence of traditional milk and milk products. Therefore in order to ensure that consumers can make conscious choices, transparent and accurate labelling of goat milk products is recommended in relation to the geographical origin of the products and the substances used in the products (Bhattarai, 2012). Globally, mislabelling of dairy products has been documented. For example, it is recorded in Paraiba State, Brazil, that bovine milk is somewhat present in goat milk provided by smallholders (Di Pinto et. al., 2017). Later, in Rio de Janeiro's largest metropolitan region, it was shown that 20 out of 20 local goat cheese products were contaminated with cow's milk, even though the labels did not indicate the addition of cow's milk (Rodrigues et. al., 2012). Furthermore, there is no recent report on the authenticity of goat milk in Malaysia; it is therefore important to conduct a study on this matter.

This study aims to detect the authenticity of self-milking of raw milk from local farms and local markets in every state in Malaysia, taking into account the importance of unique characteristics of goat milk to consumers and that the authenticity of food components is one of the critical elements of food safety and quality. Cytochrome b (cyt b) has been shown to be an important molecular marker to investigate the molecular relationship between raw goat milk self-milking and commercialised goat milk. In our research, using MEGA X tools, we conducted phylogenetic analysis to get deeper into the relationship between raw and commercialised goat milk.

METHODS

Collection for Raw and Commercialized Goat's Milk

Between November and December 2018, raw goat milk was individually milked from local goat farms representing *Capra aegagrus hircus* (Jamnapari) from 14 Malaysian states (Perlis, Penang, Kedah, Perak, Kelantan, Pahang, Selangor, Negeri Sembilan, Kuala Lumpur, Melaka, Johor, Sabah and Sarawak). The samples were obtained by direct milking in sterile bottles and kept in an ice box containing ice. Under laboratory conditions, the samples were stored at -20 ° C and 95 per cent ethanol at ambient temperature when in the field.

The Nubian brand and UK Farm brand were collected for both brands from the North, East, South West, and Klang Valley of Malaysia. The range of products from commercial brands was focused on their presence in Malaysia's hypermarket chains (AEON Jusco, AEON Major, Tesco, and Jaya Grocer). Between November and December 2018, the purchase of commercialised goat milk was also reported in hypermarkets. Commercialized milk samples were bought in packets of 250 ml and samples were processed at -20 °C before study.

Genomic Extraction

Following the manufacturer's (Promega, USA) protocol description, genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit. In the extraction buffer in mL tubes, the milk samples was homogenised. Then, the tubes were centrifuged. To remove the remaining RNA from the sample, a buffer containing digested tissue and DNA was relocated to a new tube. To extract the RNA contamination, 3 µL of Rnase (10 mg/mL, Promega) was applied to the DNA dissolved in the TE Buffer (Tris-EDTA, pH = 8.0). The tubes were incubated for 3 hours at 37 C. At 4 C for future application, the excess DNA is processed.

Sequence Analysis

For all the goat's milk samples of *Capra aegagrus hircus* and *Ovis aries*, data analysis was conducted. Using Clustal X, which is a programme that performs optimum sequence allignment, multiple sequence allignment was carried out.

Phylogenetic Analysis

The DNA sequence data for all samples were analysed for ambiguities and the nucleotide sequences obtained were aligned by Clustal X with default settings (Golinelli et. al., 2014). *Ovis aries* is used as the outgroups when constructing the phylogenetic tree. Since the variation within the nuclear loci is low, Neighbor-Joining (NJ), Maximum Parsimony (MP), Molecular Evolution (ME) methods are used to determine the relationships between sequences for each locus using the program MEGA X (Larkin et. al., 2007). Support for nodes was estimated with 500 replicates in the bootstrap technique. Bootstrap method is an efficient way to estimate reliability of phylogenetic tree.

RESULTS

Phylogenetic Analysis

The cytochrome b was partially sequenced from 26 samples. Phylogenetic trees are reconstructed using the NJ (figure1), ME (figure 2), MP (figure 3) methods. Overall, all the trees method showed similar pattern. *Ovis aries* was used as the outgroups.

One of the method of the evolutionary history was inferred using the Neighbour-Joining method (Kumar et. al., 2018). The bootstrap consensus NJ tree evolutionary relationship determined from 500 replicates to represent the evolutionary history of the taxa analysed. The tree showed the optimum tree with the sum of branch length = 8.47836804. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Saitou & Nei, 1987) and are in the units of the number of base substitutions per site. This analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pair-wise deletion option). There were a total of 2912 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Larkin et. al., 2007).

The other method is Minimum Evolution method (Tamura et. al., 2004). The bootstrap consensus ME method showed the optimal tree with the sum of branch length = 8.44293848 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Saitou & Nei, 1987) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 1. The Neighbor-joining algorithm (Kumar et. al., 2018) was used to generate the initial tree. This analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pair-wise deletion option). There were a total of 2912 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Larkin et. al., 2007).

For the Maximum Parsimony method, the most parsimonious tree with the length = 7768 is shown. The consistency index is 0.567714 (0.539495), the retention index is 0.581140 (0.581140), and the composite index is 0.329921 (0.313522) for all sites and parsimony-informative sites (in parentheses). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (pg. 126 in ref. [Rhetsky & Nei, 1992]) with search level 0 in which the initial trees were obtained by the random addition of sequences (10 replicates). This analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 2912 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Larkin et. al., 2007). Phylogenetic trees for all the three methods are shown in the figures below, in which blue square indicates fresh samples, red square indicates commercialized samples and *Ovis aries* is used as the outgroup.

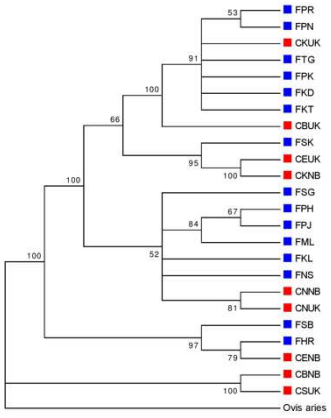

Figure 10. NJ Tree with bootstrap of 500 replicates.

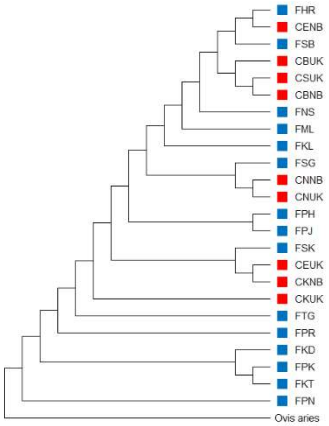

Figure 2. MP Tree with bootstrap of 500 replicates.

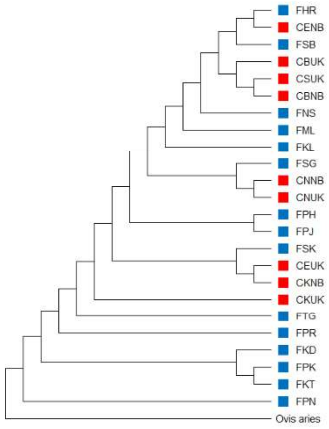

Figure 3. ME Tree with bootstrap of 500 replicates.

Content Analysis

In the holy Quran and Hadith, goat milk is mentioned many times directly (hakiki) or in terms milk in general . In Surah Al-Nahl, verse 66, Allah said;

وَإِنَّ لَكُمْ فِي الْأَنْعَٰمِ لَعِبْرَةً ۖ نُّسْقِيكُمْ مِّمَّا فِي بُطُونِهِ مِنْ بَيْنِ فَرْثٍ وَدَمٍ لَّبَنًا خَالِصًا سَائِغًا لِّلشَّارِبِينَ (٦٦)

"And there is certainly a lesson for you in cattle: We give you to drink of what is in their bellies, from between digested food and blood: pure milk, pleasant to drink" (66).

In this verse, firstly Allah stated that there is many lessons that we, humans, can learn from farm animals. In fact, many benefits can be studied and obtained based on the knowledge of natural milk production and the milk's contents. Indeed, Allah created His creation perfectly (Machrus, 2017).

According to Tafsir Al-Misbah (Indonesian Translation), the phrase 'between digested food and blood', which refers to the natural milk production in the animals, the word 'between' is not specifically referred to place or location, but to clarify that milk is not the same as blood because blood flows through blood vessels and milk does not. In addition, milk is free from toxic and waste substances, packed with nutrients, and beneficial. On the other side, the phrase "pleasant to drink", based on Tafsir al-Thabari (Indonesian Translation), means milk contains complete composition and characteristics in terms of its chemical amd physical properties.

In various study, several researchers have attempted to use phylogenetic analysis. Phylogeny analyses based on DNA sequencing were performed using MEGA software in a study on goat breeds in Jordan and showed a fairly evolutionary distinction between goat breeds in Jordan. In order to provide more evidence, the phylogenetic tree is reconstructed to demonstrate a near phylogenetic alliance between the breeds (Raed et. al.m 2012). The use of phylogenetic tree and sequencing to explain evolutionary and phylogenetic genetics in any breed is therefore efficient and accurate, as shown by the study in reference (Raed et. al., 2012). Phylogenic research also helps to determine whether DNA is the cause of the strain. In a research by Ayyez, 2017, for example, a molecular identification and phylogenetic analysis was undertaken due to the spreading of Coxiella burnetti infections across the globe. In Al Diwayana City, Iraq, samples of local goat milk were collected to be examined and tested. As a result, the amplicon sequences of the strain showed a close relationship between the NCBI-Blast Coxiella burnetti transposase gene from India and Brazil and the NCBI-Blast Coxiella burnetti transposase gene from the United States, Portugal and Taiwan (Ayyez, 2017).

DISCUSSION

Genomic Extraction

DNA-based methods have been commonly used in recent years for species recognition of milk obtained from somatic cells of milk or milk-deprived products (Lipkin et. al., 1993). Protein-based techniques are seldom used and can fail because treatments at high temperatures may cause excessive protein-induced proteolysis (Reale et. al., 2008). The DNA profile of *Capra aegagrus hircus* was determined in our sample using the method of DNA extraction (Wizard Genomic DNA Purification Kit). A total of 25 individual genomic DNA was collected from the milk samples. It had to be the largest molecular weight marker on the ladder in order to classify the genomic DNA. Nucleic acid was used in identification assays because compared to nuclear DNA, they provide higher levels of mt-DNA copies per cell to maximise and increase the success of DNA extraction. As a result, both milk samples from raw milk and commercialised milk containing DNA have been shown in this experiment, so the samples can be used for further studies.

Phylogenetic Analysis

With advances in molecular biology and DNA sequencing methods, it is easier to classify the genomes of different species easily. This DNA sequence analyses provide valuable knowledge about their taxonomy, gene makeup, and utilizes. A primary marker that helps researchers to recognise and classify *Capra aegagrus hircus* species for the many benefits it provides is mitochondrial DNA. DNA sequence polymorphism of commercialised and raw goat's milk was analysed in this analysis to explain the variations between the sequences in separate methods with the help of phylogenetic tree. Therefore, it is possible to determine the authenticity of the commercialised goat's milk.

The neighbor-joining method is part of the distance-based method, which minimises the overall length branch of each Operational Taxanomic Units (OTU) clustering point. Meanwhile the character-based Maximum Parsimony method

minimises the cumulative number of evolutionary steps expected to describe a given collection of data allocated to conclude a phylogenetic tree. The Minimal Evolution method is based on the idea that the most likely tree to be valid is the tree with the smallest sum of branch lengths. Distance checks that are accurate at the same positions on repeated hits are used.

The congruent findings of the trees for *Capra aegagrus hircus* in our research indicate that the commercialised samples of goat's milk are considered to be authentic. The phylogenetic trees shown using analyses of MP, NJ, and ME suggest that *Capra aegagrus hircus* formed a monophyletic clade or taxa with a common ancestor in both commercialised and raw samples. The sequences are related phylogenetically to each other. The molecular data indicates that raw samples have no common distance between them. A strong difference in the sequence of *Capra aegagrus hircus* and *Ovis aries* based on the phylogenetic trees proved that there is no adulteration of sheep's milk in the commercialised goat's milk in Malaysia.

By using the cytochrome b marker, the analysis revealed a remarkable degree of similarity between the raw and commercialised samples of *Capra aegagrus hircus*. A similar taxon studied in the cytochrome b molecular marker is phylogenetic inference through neighbor-joining, minimum-evolution, and maximum-parsimony analysis revealed by goat milk samples.

CONCLUSION

This research has supported significant molecular analysis based on the species *Capra aegagrus hircus* to identify the validity of goat milk in commercialised goods in Malaysia. It has shown that in the sold samples there is no addition and adulteration has happened, making them better to be bought by consumers to get the full value of goat's milk. In order to find the genetic differences between them the phylogenetic tree is reconstructed for all samples and *Ovis aries* was used as the outgroup. The DNA was first sequenced using Clustal X software. Using MEGA X software, three tree reconstruction techniques, Neighbor-Joining, Maximum Parsimony and Molecular Evolution, were performed and compared. The data revealed that raw and commercialised samples with a similar ancestor formed a monophyletic clade or taxa. This outcome suggests that the DNA sequences of the raw and commercialised samples are identical.Finally, goat milk packaged and marketed to consumers can thus be trusted and consumed without thinking about the occurrence of allergic reactions, high sugar content and other undesirable factors. In the Quran, Allah stated the benefits and explain the process of milk production that occurred naturally and how humans can take lessons from the event itself. Moreover, in various stiudies, phylogenetic analysis is used widely.

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