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A Molecular Technique for Detecting Cow's Dried and Liquid Packaged Milk products Adulteration

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ABSTRACT

The authenticity of animal-based products is a significant concern when it comes to protecting consumer rights and ensuring accurate product labeling. Molecular techniques, such as DNA barcoding using the cytochrome b gene (Cyt b), are powerful tools for species identification. Our research addresses knowledge gaps in the authentication of cow's dried powder and liquid packaged milk. By analyzing genetic sequences, we have been able to detect genetic variations and construct phylogenetic trees to differentiate cow's milk from other species. DNA was extracted, and a specific region of the cytochrome b (Cyt b) gene, a 359 base pair was amplified. The amplified DNA fragments were then sequenced. The obtained Cyt b sequences were aligned with a reference sequence using Clustal Omega software and further refined using Jalview software. Our analysis did not reveal any significant differences in the ratio of transitions to transversions (Ts:Tv) among the sequences. Consequently, the milk samples from the Kurdistan region formed a distinct, single-origin group (monophyletic clade) in the phylogenetic tree with reference sequence (OQ535548.1) with high identic values (100%). The Cyt b sequences of the samples were submitted to GenBank with accession numbers PP957613 to PP957618. The base composition of the Cyt b gene sequence had a higher AT content (59%) than GC content (41%). These results positively dispelling the concern regarding the authenticity of dried milk powder and liquid packaged milk.

1.Introduction

The authenticity of animal products has become a major issue in ascertaining consumers' rights with ensuring accurate product labeling (Ballin, 2010). Such labelling should provide comprehensive information regarding composition, ingredients, production technology, genetic identity and possible allergic pathologies that may occur (Taha, 2022, Meijer et al., 2021). Several animal species such as cattle, buffalo, goat and sheep provide milk for dairy products. Then dairy products represent a large proportion of the food industry (Maudet and Taberlet, 2001), However, due to the relatively high cost of milk, dairy products are often susceptible to adulteration practices, particularly in the case of milk and its derivatives (Sakaridis et al., 2013).

Therefore, species identification of milk and dairy products is of great concern for Protection against species substitution or admixture in dairy products (Abdelfatah and El-Araby, 2015). Common adulterations of dairy products are the substitution of higher value milk by cheaper one (Zachar et al., 2011). Cow's milk may be used for adulteration of buffalo's milk due to its lower cost and larger availability deriving from greater productions, compared to buffalos in the same farm (Poonia et al., 2017) Nevertheless, this practice should be avoided due to several health concerns. Cow's milk protein can be a significant allergen for some individuals, even in low concentrations, and it has been identified as a primary cause of adverse dairy-related reactions (Nehra et al., 2020). Cow's milk also may be avoided due to religious, ethical or cultural objections (Shatenstein and Ghadirian, 1998). Moreover, the adulteration of other milk with cow's milk may be considered as a health risk in current food safety requirement.

In recent years, molecular approaches based on PCR have revolutionized biological sciences by enabling the development of rapid, sensitive, and specific methodologies. (Jalalizand et al., 2012). These techniques are particularly valuable when traditional methods are limited or inconclusive. (Valenzuela et al., 2007). The mt cyt b, mt COI, mt COII, and nuclear EF1 DNA sequences for key species identification that have been gathered through numerous molecular phylogenetic investigations (von Dohlen et al., 2006, Kim et al., 2010, Muhammad et al., 2022). Within mt-DNA, mitochondrial cytochrome b gene (mt cyt b) encodes a famous protein that creates complex III of the mitochondrial phosphorylation system and is best single known encoded by the mitochondrial genome (Tao et al., 2014).

It has been argued for now, many claim that milk powder sold in the Kurdistan Region of Iraq are often adulterated with non-dairy substances and packaged liquid milk labeled as pure cow's milk, claims of adulteration with other sources. This practice is culturally unacceptable and has not been extensively studied using molecular techniques. To address this issue, we aim to employ DNA sequence analysis and alignment of the mitochondrial cytochrome b gene to detect fraud in various brands of milk powder and packaged liquid milk in Erbil Kurdistan region.

2. Materials and Methods

2.1 Sample Collection, Preparation and DNA Isolation

In this study, we used 6 milk brands, from these brands we collected 54 milk samples (27 dried powder '3 brands' and 27 liquid packaged '3 brands') in Erbil Province, Kurdistan Region-Iraq, during February-April 2024. These samples were randomly selected from various markets and stored refrigerated to prevent contamination. Six pooled samples were prepared (3 dried powder and 3 liquid packaged) were chosen for further testing. Then taken to the private molecular laboratory in Erbil, then DNA isolation was carried out. For the DNA extraction process, only the somatic cells were used after centrifugation. Total genomic DNA was extracted following the instructions of the commercial kit Beta Bayern tissue DNA preparation Kit (Beta Bayern GmbH .90453 Bayern, Germany) DNA extraction. Then the isolated DNA was kept at -20°C for the downstream applications for a week. The quality and quantity of the isolated DNA checked by Nanodrop 1000 (Thermo scientific UK), which were ranged from 1.7 to 1.9.

2.2 PCR Primers

The primers of specific gene in mitochondria which is known as mt-Cyb gene was chosen for PCR amplification, using the following pairs of primer that is shown in (Table 1) which exemplified the specific data on the Mt-primers (Khan et al., 2018). The Cytb primers were synthesized in South Korea by a company of Micro-gene.

Table 1: PCR primer Cyt b gene used for molecular study of milk adulteration

Gene name	Nucleotide Sequences	amplified size	Reference
Cyt b (NP_904340.1, gene ID: Gene ID: 17711)	F:5'CCATCCAACATCTCAGCATGATGA AA-3'	359 bp	Khan *et al* (2018)
	R:5'GCCCCTCAGAATGATATTTGTCCTCA-3'		

2.3 PCR Amplification

Polymerase chain Reaction (PCR) was carried out in a private laboratory in Erbil using a thermal cycler (Bioresearch PTC-200 Gradient thermocycler) in a final amplification volume for reaction mixture was done in 50µl as shown in (Table 2). The PCR program was adjusted according to the selected primer by applying a modification program as in (Table 3). The amplicon size was 359 bps. Amplicons were visualized in a 1.5% agarose gel electrophoresis (45 seconds, 75V 1X TBE buffer) that is stained with 5 µl EtBr (LOT:110802BB197, Bio Basic Inc.) and visualized under UV Transilluminator (Biostep-UST-20M-8K). Then the PCR products were kept at -20°C for the future uses.

Table 2: PCR amplification components

No.	PCR components	Concentration	Volume (µl)
1	Master Mix (AMPLIQON A/S Stenhuggervej 22)	2x	25
2	Forward Primer	20 Pmol	2
3	Reverse Primer	20 Pmol	2
4	Template DNA	50ng/µl	5
5	DNase free Water	-	16
Total			50

Table 3: PCR program for Cyt b gene

Step	PCR temp. (◦C)	Time (min.)	Cycles
Initial denaturation	95	5	1x
Denaturation	95	40 sec	35x
Annealing	58	40 sec	
Extension	72	1	
Final extension	72	5	1x
Storage	4	∞	-

2.4 DNA Sequencing and submission

The PCR product was labelled with a unique identifier, including the sender's information then packaged well in a container designed for biological samples that prevents breakage and temperature fluctuations that kept cold then sent the samples to Korea for sequencing. The Senger sequencing of partial gene of Mt-DNA-Cyt b was done at the Micro-gene Center in Korea via BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems). The product was evaluated utilizing an ABI PRISM 310 (PE Applied Biosystems). The Mt-Cyt b gene sequence chromatograms produced in the current work were collected and by hand edited and inspected through FinchTV v1.4 (available at https://digitalworldbiology.com/FinchTV. Then the attained sequence has been submitted and confirmed on NCBI nucleotide database, which is available in (https://blast.ncbi.nlm.nih.gov/Blast.cgi), in GenBank and received the accession numbers (PP957613 - PP957618) for Cyt b gene for comparison and alignments query sequence with downloaded biological sequences (OQ535548.1) to realize the source of the milk samples.

2.5. Alignment and Phylogenetic

To achieve sequences of only one consensus, chromatogram was examined and the DNA sequence alignment was carried out through Clustal Omega (available at https://www.ebi.ac.uk/Tools/msa/clustalo/. The phylogenic tree was created via the Maximum Likelihood method grounded on the Tamura-Nei

model in MEGA11 software (Tamura et al., 2021), with the available sequence of the Cyt b gene of the cow, was downloaded from the NCBI nucleotide for the phylogenetic analysis.

3. Results

3.1. DNA Extraction and PCR amplification

Based on the proposed methods 20-25µg of genomic DNA included Mt-DNA was successfully isolated from the milk samples, with the purity was 1.7-1.9. Then 6 pulled sample were prepared for the downstream application (each pooled sample consisted of nine milk samples). After then, the amplification was carried out by traditional PCR with the help of pair of primers for the Mt Cyt b gene (NP_904340.1, gene ID: Gene ID: 17711), the amplicon size was 359bps and bands were separated by 1.5% agarose gel electrophoresis (45 seconds, 75V 1X TBE buffer) stained with 5µl Ethidium Bromide (LOT:110802BB197, Bio Basic Inc.) visualized under UV Transilluminator (Biostep-UST-20M-8K) as shown in (Figure 1), The 359bp fragment of the cytochrome b gene was reported to be highly polymorphic and could be used to differentiate milk samples in different pieces (Taha, 2022).

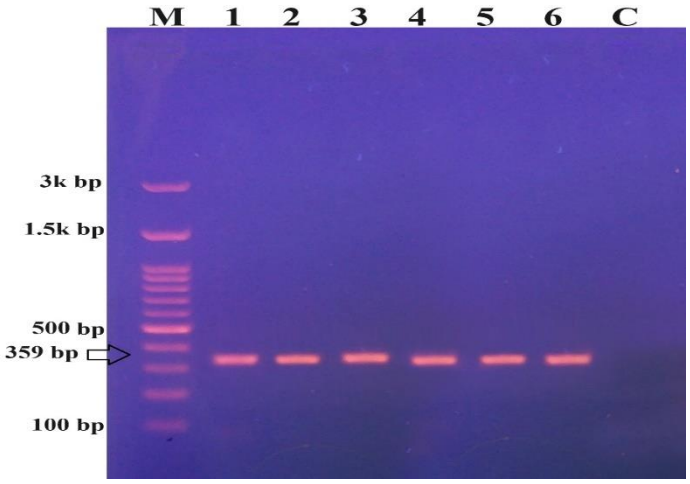

Figure 1: PCR amplification of partial cytochrome b gene from milk. M; indicate: ladder 100-3000 bp (Cat NO. DM012-R500, Promega- USA), lane 1-6: 359 bp of PCR products from dried milk powder and liquid packaged milk and C is negative control.

3.2. Sequence Analysis

The PCR product kept and sent it to do partial Senger sequencing of Mt-DNA cyt b gene, by ABI 3130X genetic analyzer (Applied Biosystem), then the sequence nucleotides submitted and confirmed on NCBI nucleotide database with the accession numbers (PP957613- PP957618). The nucleotide frequencies Mt-DNA Cyt b gene sequence of milk samples was A 94 nt (31%), T 82 nt (29 %), C 76 nt (25 %) and G 45 nt (15 %), while the base composition of the cyt b gene fragment created 59 and 41% A-T and G-C% respectively

3.3. Alignments and Variation Analyses for the milk samples

A query sequence is aligned based on their protein sequence that translated by MEGA V.11 software, with a reference sequence (OQ535548.1) to reveal the similarity and genetic variations between them as shown in the (Figure 2), alignment has been done by (MSA Clustal Omega), (available at https://www.ebi.ac.uk/Tools/msa/clustalo/ and used Jalview software to visualize and edit sequence alignments (Same color indicated perfectly matched sequences, * missing amino acids, X mismatches and – is gaps). As we can see here the query sequence has the same sequence if we compare with a reference database sequences (OQ535548.1).

The alignment indicated that there is no mutation happened in the sequence of the milk samples, so there are full matched 100% with a reference sequence. As well as the Percent Identity Matrix created by Clustal2.1 as shown (100%) all the sample with each other and the samples with the reference sequence. The base substitution of the sequences in this study (transition and transversion) are equal to zero.

The results from submission to the NCBI GenBank indicated that the highest query cover was 100% with (reference sequence) with 100% identic number. Since there were no previous submission about cyt b gene of milk in Iraq and Kurdistan for specific, we see that our sequence submission revealed the first record in NCBI GenBank and indicated the first record.

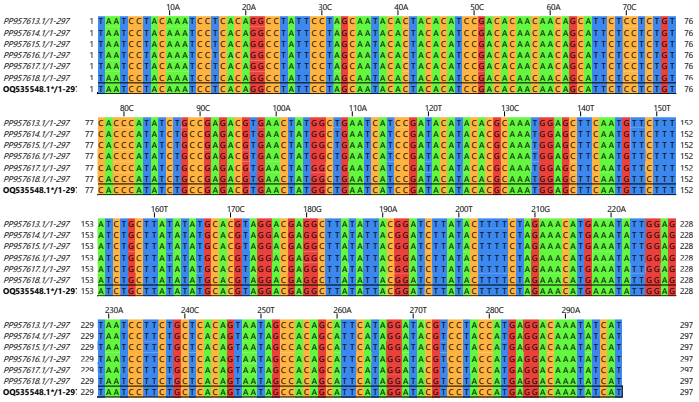

Figure 2: Clustal Omega multiple sequence alignment of the cytochrome b gene of 6 milk samples and reference sequence*.

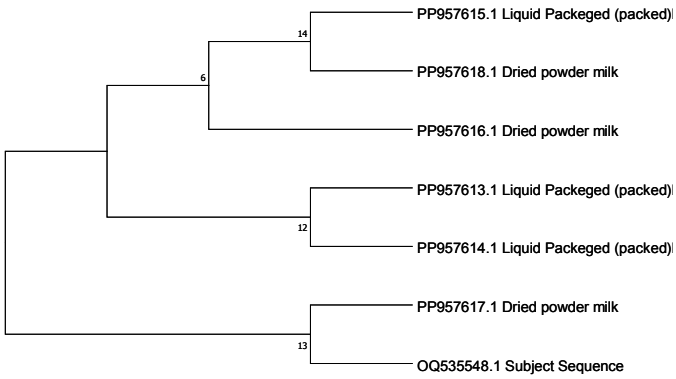

Figure 3: Phylogenic tree of cyt b gene of 6 Milk sample and a subject sequence (as a reference).

3.4. Phylogenetic Analysis of Mt-Cyt b gene of milk

The bootstrap method for test of phylogenic tree was created with No. bootstrap replication is 1000 via the Maximum Likelihood statistical method grounded on the Tamura-Nei model with very strong branch swap filter in MEGA11 software (Figure 3). A segment of the mt- cyt b gene has utilized as an species DNA barcode identification, it is understood that mutation rate occur fast and it is adequate to differentiate even between closely related species (Hebert et al., 2003).

To do phylogenetic analysis, we took a reference sequence in the same species. The constructed tree in this study indicated the formation of a clade depending on variations in cyt b gene sequences. Maximum Likelihood related tree established the close relationships achieved from BLASTn alignment, displayed that the query sequence clustered within the same clade with prior recorded sequence that analysis within the same species in other country. From sequence divergence was exposed that species fitting to corresponding genera were adjoining to one. Moreover, the query sequences in the Kurdistan region of Iraq along with the (OQ535548.1) sequence was clustered together with one common ancestor.

4. Discussions

This study was initiated to address public concerns regarding the origins and authenticity of these milk products. By employing molecular techniques, we aimed to provide accurate and reliable information to dispel these doubts. The result provided partially forward sequencing of mitochondrial cyt b gene for milk samples for the first time in Kurdistan. The obtained sequences were submitted to the GenBank and received their accession number (PP957613 - PP957618) for verification, so as to be a database for the upcoming studies in the future. Moreover, multiple sequence alignments were applied grounded on the downloaded reference sequences (OQ535548.1) coming from the species which were already reliably identified. In our investigation, the query sequence we align 100% with the reference sequence (OQ535548.1), so that without doubt this leads to show full similarity between query and reference sequences. Then the genetic variations both (transition and transversion) have not occurred and the mutation rate equal to zero, which illustrated in the (Figure 2). Although various factors may contribute to the debate surrounding these milk products, one of the most significant issues is the difficulty in dissolving some samples, such as PP957618, in warm water, making them unsuitable for yogurt production, this is agreed with Paul et al. (2023) that showed the heat treatments applied in each step of dried milk can promote protein degradation, such as protein denaturation, aggregation, or

modification, which could be linked to a deterioration in powder solubility in dairy powder products, such as solubility and other physical properties. However, these are may due to the way that they used to produce these dried powders which is a processed product that has undergone heat treatment, skips the fermentation step, leaving it a simple milk concentrate without the desired yogurt attributes, and in essence, while dried milk powder is a milk-based product, it's missing the vital ingredients and processes necessary to create yogurt. Regarding the physical properties, they are crucial for managing dairy powder products during the final drying process and for their use as food ingredients (Rosa and Prudencio, 2023). In addition, recent data show that protein denaturation and modification that occur during powder manufacture could promote a deterioration in the product digestibility and, as a result, a loss of its physiological benefit (van Lieshout et al., 2020).

The developed cyt b sequences have a strong base composition bias of both A/T (51%), G/C (31%), this is consistent with previous findings on mitochondrial sequences used for species identification. (Wang et al., 2017, Khdr et al., 2020) The tendency near this bias might be due to either the fact that G/C bonds are stronger with three (H-bonds) than A/T Tow (H-bonds) or might be by the chance via natural selection for boing adapted with the natural disasters (Wang et al., 2013).

We present the first cyt b gene phylogeny for milk adulteration to evaluate relationships among the major grouping. Monophyly was recovered in all samples with reference sequence. Our findings relatively consistent with (El-Rady and Sayed, 2006). However, several challenges hindered progress, including the difficulty in obtaining detailed information about sample origins due to labeling limitations. Additionally, the lack of adequate funding and bioinformatics resources, coupled with limited expertise in this field, further constrained the scope of this research.

Conclusions

This study pioneered the use of molecular techniques in Kurdistan to detect milk adulteration. By sequencing the mitochondrial cytochrome b (mt-cyt b) gene, we effectively aligned query sequences with closely related species, identifying regions of similarity and genetic variation. Phylogenetic analysis, based on mt-cyt b gene sequences, clustered milk samples from similar genera, demonstrating its suitability for species identification. Future research should expand sample size and geographic scope to create a comprehensive phylogenetic profile for milk species identification.

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