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RESEARCH ARTICLE

# ITS2 secondary structure data improves authentication of *Moringa oleifera* tea products when using with DNA barcoding

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## Abstract

Adulteration is a severe issue affecting the herbal industry. Therefore, a robust tool is needed to address this problem. In the current study, Moringa-based products (tea) authentication was conducted using DNA barcoding. Two different barcode regions, rbcL and ITS2, were investigated in terms of their effectiveness in authenticating the herbal products. To proceed with the DNA barcoding, a good quality of gDNA is a prerequisite for PCR amplification. Hence, two lysis buffers, PL1 and PL2, were compared to obtain good quality gDNA. Results revealed that a higher gDNA yield was obtained from the fresh plants using PL2, but a lower gDNA yield was obtained for the tea products except for sample P3. The PCR reaction was successfully conducted by amplifying two different barcodes, rbcL and ITS2. The amplicon size for the fresh plant was 643 bp for rbcL and 404 bp for ITS2. In contrast, the generated rbcL amplicon sizes for herbal tea products were 672 bp for P1, 679 bp for P2, 679 bp for P3, and 674 bp for P4. For rbcL and ITS2 amplicon sizes were 395 bp for P1, 406 bp for P2, 398 bp for P3, and 413 bp for P4. The amplified PCR products were analyzed using bioinformatic tools. The neighbour-joining (NJ) analysis for the rbcL barcode indicated that the P2, P3, and P4 tea products were categorized in the same clade with the *M. oleifera* sequence obtained from GenBank. Simultaneously, P1 was clustered individually with other closely related species. The analysis for the ITS2 barcode showed that all samples were grouped in the same clade. Incorporating secondary structure prediction after NJ analysis improved the discrimination between species.

## Keywords

DNA barcoding; Moringa Tea Product; rbcL; ITS2; Secondary Structure

## Introduction

The application of herbal materials for healthcare and therapeutic purposes has been practiced for centuries. This has led to a boom in the usage of herbal products worldwide, including in Malaysia. The trade of herbal materials in healthcare industries obtains a high profit, and this can be proven when the global sales of herbal products are estimated to reach USD$140 billion by 2024 (1). The World Health Organization (WHO) has stated that the trade in medicinal plants, herbal raw materials, and herbal drugs is growing at an annual rate of about 15% (2). Malaysia, in particular, is rich in up to 2,000 herbaceous species found in Peninsular Malaysia, Sabah, and Sarawak (3). The domestication of wild herbs has increased the production of herbal medicinal products in Malaysia. Additionally, the increasing trend

of returning to nature and concern over adverse reactions from Western medicines may be the contributing factors to the rise in the usage of herb-based products. Consumers accept herbal medicine because they believe that herbal products are natural, affordable, readily available, and safe, and perceive them to have specific health benefits (4). However, fraudulent activities in the production of herbal products can occur either intentionally or unintentionally.

Therefore, a reliable authentication method is needed for the herbal industry and regulatory agencies to ensure consumer safety since there is an increase in the global trading of raw and processed herbal products (5). Several methods have been implemented in plant identification, such as the traditional methods including organoleptic methods (i.e., identification by the senses including taste, sight, smell, and touch), macroscopic and microscopic methods (i.e., identification by shape, colour, and texture), and chemical profiling (e.g., thin-layer chromatography (TLC), high-performance liquid chromatography-ultra violet (HPLC-UV), and high-performance liquid chromatography-mass spectrometry (HPLC-MS). However, none of these methods can easily distinguish the related species in processed products (6). This is due to the fact that the traditional method requires a skillful person for macroscopic and microscopic examinations, while the chemical profiles or markers in the chemical profiling method may be affected by physiological and storage conditions (7). Additionally, there is unavailability of any single biomarkers that can be assigned toeach species. Therefore, the authentication of herbal plants at the DNA level by molecular markers is a potential tool due to the stability of DNA, which is not affected by external factors and is found in all tissues (8).

Phylogenetic analyses of ITS2 DNA barcoding typically rely solely on its nucleotide sequences. However, several studies have revealed that incorporating the secondary structure enhances the authentication of herbal medicinal products, Ayurvedic medicine, and traditional Chinese medicine (9), (10), (11), (12). The utilization of the secondary structure demonstrates the crucial potential of DNA barcoding in authenticating genuine herbal products. Phylogenetic analyses often rely on nucleotide similarity for homologous alignments, but the alignment fidelity decreases beyond the genus rank due to excessive sequence variability. The ITS2 secondary structure offers a solution to this challenge. Conserved nucleotide motifs help anchor multiple sequence alignments, producing a more accurate depiction of relationships at higher taxonomic levels (13). Although ITS exhibits high nucleotide variation, the conserved region of the secondary structure can align divergent sequences beyond the genus level. As a result, ITS2 is considered a dual-purpose tool for making eukaryotic evolutionary comparisons (14). Previous research has demonstrated that RNA secondary structure prediction is a powerful method for species identification. For both fresh plant and herbal product samples, 400 µL of cetyltrimethylammonium bromide (CTAB) buffer (PL1) and 10 µL RNase A were added, gently vortexed, and incubated for 10 minutes at 65°C. Similarly, for sodium dodecyl sulfate buffer (PL2), 300 µL was used, and 10 µL RNase A was added, gently vortexed, and incubated for 10 minutes at 65°C. The inclusion of secondary structural information also enhances species identification resolution in other plant species, as RNA secondary structure prediction plays a significant role in representing the function of rRNA and tRNA in the conserved ITS rRNA region (15).

Herbal tea and green tea are widely consumed worldwide due to their believed beneficial health effects and high antioxidant content. Malaysia, being rich in various herbal resources that can be transformed into beverage products (i.e., tea, coffee, and herbal drinks), has seen many beverage manufacturers launching these products in the market to promote immune systems boosting among consumers. This has led to the growth of the herbal tea industry in Malaysia , including the production of herbal products based on *Moringa oleifera*. In Malaysia, *M. oleifera* is known as *merunggai* or *rambungai*, and it is considered a potential medicinal plant for treating various diseases because of it's nutritive and pharmacological potentials such as antimicrobial, anticancer, antihyperlipidemic, antidiabetic, antiulcer, analgesic, antifertility, anticonvulsant, and hepatoprotective (16). The medicinal values of this herbal plant are well known as it has been used in the herbal medicinal practices of the Indians and Africans since ancient times (17). *M. oleifera* is also rich in vitamins, proteins, and minerals and has high nutritional value. Therefore, it has been utilized as a dietary supplement (18). The herbal tea of *M. oleifera* is also believed to be rich in phenolic compounds and antioxidants (19). Consequently, herbal products based on *M. oleifera* have been commercialized in the forms of teabag, dried leaves, capsules, tablets, liquid, powder, therapy oil, and premix coffee. However, the products are susceptible to fraudulent activities, which could potentially harm the consumers. Therefore, a fast and reliable authentication method for quality control to ensure only genuine *M. oleifera* raw materials are used in the production of herbal tea products. In current study, DNA barcoding analysis was employed to identify the correct raw materials of *M. oleifera* in the production of herbal tea products.

## Materials and Methods

The leaves of *M. oleifera* were collected from a nearby residential area at Taman Sri Pulai Perdana, Johor Bharu, and abbreviated as FP A (first compound leaves from the tip of the stem), FP B (second), FP C (third upper), and FP D (third below) as shown in Figure 1 (A). According to a previous study, fresh and young leaf materials were identified as the best sources for obtaining good quality DNA due to their lower content of polysaccharides compared to mature leaves, which contain high amounts of both polyphenols and polysaccharides that can pose challenges during DNA isolation (20). Additionally, four different Moringa tea herbal-based products, labeled as P1-P4 in Figure 1 (B), were purchased online and included in the present study.

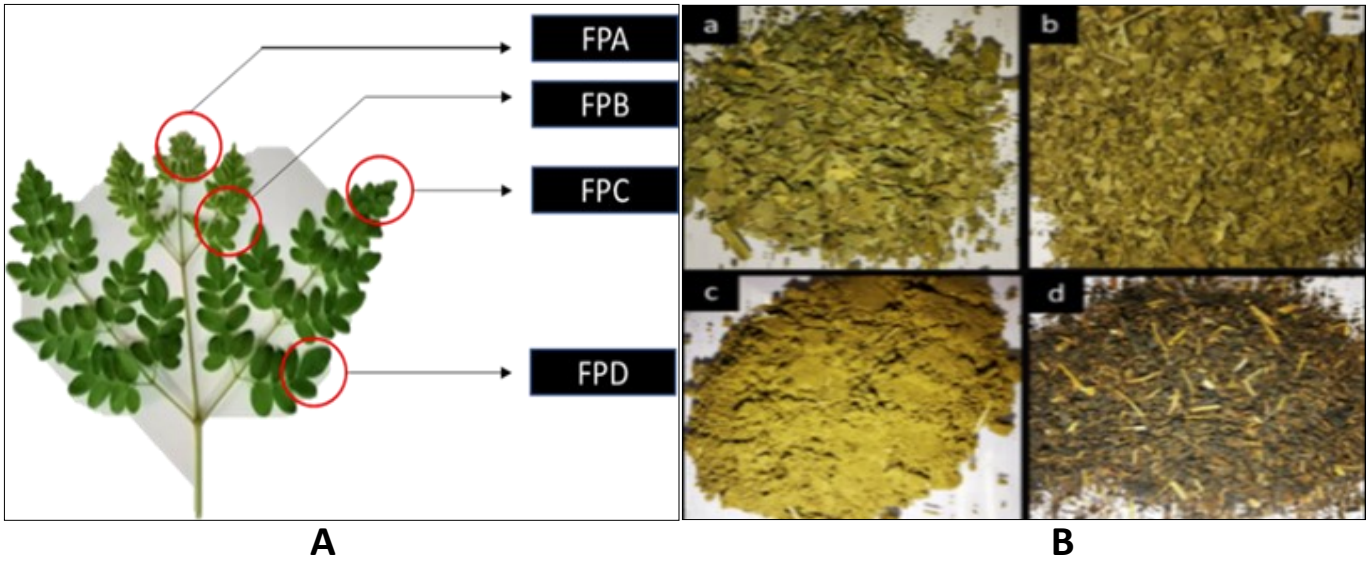


**Fig. 1.** (**A**) Parts of compound leaves of *M. oleifera* and (**B**) tea products of Moringa used in the present study (**a**) Product 1 (P1), (**b**) Product 2 (P2), (**c**) Product 3 (P3), and (**d**) Product 4 (P4).

### *Genomic DNA extraction*

Approximately 100 mg of *M. oleifera* leaves and 100 mg of herbal products were ground in liquid nitrogen separately and placed in a 1.5 ml sterile microcentrifuge tube. The DNA extraction was performed using NucleoSpin Plant II Kit (Macherey-Nagel, Germany), following the manufacturer's instructions and guidelines. For both fresh plant and herbal product samples, 400 µL of cetyltrimethylammonium bromide (CTAB) buffer (PL1) and 10 µL RNase A were added to the samples. The samples were gently vortexed, and incubated for 10 minutes at 65°C.

For both fresh plant and herbal product samples, 300 µL sodium dodecyl sulfate buffer (PL2),and 10 µL RNase A was added to the samples, gently vortexed, and incubated for 10 minutes at 65°C. While using PL2 buffer, after incubation step, 75 µL of sodium dodecyl sulfate buffer (PL3) was added, gently mixed, and incubated on ice for five minutes to precipitate SDS completely.

The lysate was then added to the NucleoSpin Filter column in a new 2 ml collection tube and centrifuged at 11,000 rcf for two minutes. The clear flow-through was carefully transferred to a new 1.5 ml microcentrifuge tube without disturbing the pellet formed at the bottom of the collection tube, and the NucleoSpin Filter was discarded. Next, 400 µL of binding buffer (PC), which contains 24–36 % of Guanidine hydrochloride and 35–55 % ethanol, was added to the sample and mixed by pipetted up and down five times.

The mixed solution was transferred to the Nucleospin Plant II column and centrifuged for 1 minute at 11,000 rcf. The flow-through was discarded. The washing step were initiated by adding 400 µL PW1 Buffer (36–50% guanidine hydrochloride and 36–50% isopropanol) and centrifuged at 11,000 rcf for one minute. The flow-through was then discarded. The second washing step involved adding 700 µL of wash buffer concentrate (PW2) and centrifuging at 11,000 rcf for one minute, with the flow-through was discarded. The final washing step included the addition of 200 µL PW2 buffer, followed by centrifugation for 2 minutes and the removal of flow-through. The column was placed into a new sterile microcentrifuge tube for DNA elution.

For DNA elution, 50 µL of pre-heated elution buffer (PE) containing 5 mM Tris/HCl (pH 8.5, 65°C) was added onto the column membrane surface. The column was incubated again at 65°C for five minutes and then centrifuged at 11,000 rcf for one minute to collect the eluted DNA. This elution step was repeated with another 50 µL pre-heated PE buffer (65°C) into the same tube. The eluted DNA was stored at -20°C until further use for PCR amplification.

### *Quantification of DNA*

A NanoDrop™ 1000 Spectrophotometer was used to determine the yield, and purity of genomic DNA from fresh plant and herbal products. Distilled water was initially loaded into the spectrophotometer, and 2 µL of elution buffer (PE) was set as blank. Then, 2 µL of DNA sample was loaded, and the absorbance at 260 nm was measured. The purity was evaluated by calculating the ratio of $A_{260}/A_{280}$.

For agarose gel preparation, a 1% (w/v) or 2% (w/v) agarose gel was prepared in 1X TAE buffer for the extracted genomic DNA and PCR product, respectively. After fully dissolving the agarose powder, 0.5 µL of Midori DNA stain was added. The mixture was then poured onto a casting tray with a suitable comb and allowed to solidify for approximately 30–45 minutes. Once solidified, the gel was transferred to a gel electrophoresis tank and soaked in a 1X TAE buffer solution. A 1kb DNA ladder (5 µL) (Promega, United States) was mixed with loading dye (2 µL) (Promega, United States) on a parafilm and then loaded into the first well. Approximately 200 ng genomic DNA for fresh plant and approximately 500 ng for herbal product samples were mixed with 2 µL of loading dye (Promega, United States) and loaded into their respective wells. The gel was run at 80 V for 55 minutes. Finally, the gel was observed using UV illumination of the AlphaImager® HP gel documentation system.

### *DNA barcoding*

For the PCR amplification of the ribulose bisphosphate carboxylase large chain (rbcL), a 25 µL reaction mixture was prepared. It contained approximately 100 ng of genomic DNA template from fresh *M. oleifera* plant and 60 ng for herbal products. The reaction consisted of 5 µL of 1X GoTaq® buffer, 0.5 µL of dNTP mix, 1.25 µL of each 0.5 µM forward and reverse primers, 1.5 µL of 1.5 mM of $MgCl_2$ solution, 0.125 µL of 1.25 units of GoTaq DNA polymerase, and nuclease-free water up to 25 µL. The amplification was carried out according to the PCR protocol with slight modifications: initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s for annealing, 72 °C for 60 s for elongation, and a final extension at 72 °C for 4 min.

For the amplification of the Internal Transcribed Spacer 2 (ITS2) region, PrimeSTAR® Max DNA polymerase was used instead of GoTaq® DNA polymerase enzyme. The PCR amplification was performed in 25 µL reaction mixtures containing approximately 100 ng of genomic DNA template from fresh *M. oleifera* and 60 ng from herbal products. The reaction mixture consists of 12.5 µL of 1X PrimeSTAR® Max Premix, 0.5 µL of each 0.2 µM forward and reverse primers and nuclease-free water up to 25 µL. The reactions were conducted according to a three-step PCR programs: initial denaturation at 98 °C for 10 s followed by 30 cycles at 98 °C for 10 s for denaturation, annealing at 55 °C for 15 s, and elongation at 72 °C for 5 s. The final extension was performed at 68 °C for 1 minute

The expected fragment sizes for rbcL were approximately 600-650 bp and 450–550 bp for ITS2. The specific primers used for rbcL were rbcL-F: (5'ATGTCACCACAAACAGAGACTAAAGC 3') and rbcL-R: (5' TCGCATGTACCTGCAGTAGC 3') (21). For ITS2, the primer used were ITS2-F: (5'ATGCGATACTTGGTGTGAAT 3') and ITS2-R: (5'GACGCTTCTCCAGACTACAT 3') (22). The PCR product size was validated by performing gel electrophoresis. The same procedure was followed for the electrophoresis of genomic DNA. The amplified PCR products were sent to Apical Scientific Sdn Bhd, Selangor, Malaysia for purification and sequencing services, and later analyzed and compared to GenBank databases (https://www.ncbi.nlm.nih.gov/).

### *Authentication evaluation*

Two identification methods, BLASTn, and tree-based methods were used to determine the authentication efficacy. The sequencing results were further analyzed using bioinformatics software such as BioEdit version 7.2.5.0, Jalview version 2.11.1.0, and MEGA X version 10.1. In addition, secondary structure prediction was performed using the ITS2 workbench (http://its2.bioapps.biozentrum.uniwuerzburg.de/). This complementary analysis provided valuable information on the ITS2 secondary structure, which aids in understanding phylogenetic connections within closely related datasets. The integration of secondary structure analysis strengthens the potential of ITS2 as a promising DNA barcode for Moringa, as it incorporates important secondary structure information that can improve species discrimination.

## Results and discussion

The genomic DNA extraction was successfully performed for fresh *Moringa* leaves and four selected herbal products using two lysis buffers, PL1 and PL2. The extracted gDNA with good quality was then used as a DNA template for amplifying the DNA barcodes, rbcL, and ITS2.

### *PCR amplification of DNA barcodes of M. oleifera fresh leaves*

The rbcL and ITS2 barcodes were successfully amplified from different developmental stages of leaves (FPA, FPB, FPC, and FPD). As shown in Figure 2 (lanes FPC2, FPC3, FPD1, FPD2, and FPD3), the rbcL fragments were amplified at the targeted size, approximately 650 bp, as confirmed by a previous study by Rayan (21). Similarly, the ITS2 fragments with a size of 500 bp were successfully amplified, consistent with the findings of other researchers (22) who reported a range of amplified sequence lengths from 450 bp to 550 bp for the *Moringa* plant (Figure 3). No contamination was detected, as evidenced by the absence of bands in the negative control (lane -C).

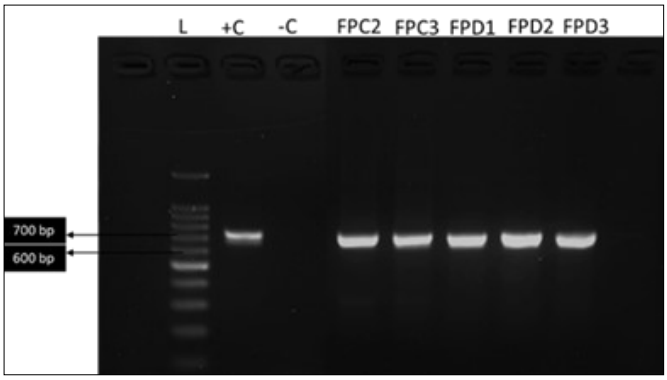


**Fig. 2.** PCR amplification for *rbc*L *M. oleifera* fresh leaves run on 2% (w/v) agarose gel electrophoresis (lane FPC2, FPC3, FPD1, FPD2, FPD3= *M. oleifera* fresh leaves). +C: *rbc*L of *L. pumila*; -C: Negative control; L: 100 bp DNA Ladder (Promega, United States)

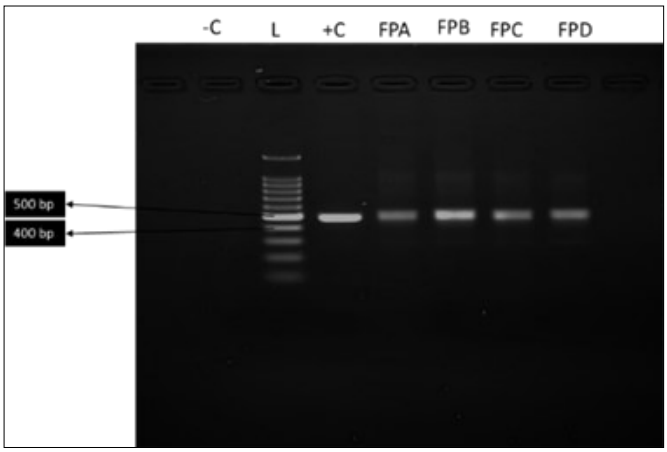


**Fig. 3.** PCR amplification for *ITS2 M. oleifera* fresh leaves run on 2% (w/v) agarose gel electrophoresis. +C: *ITS2* from *L. pumila*; -C: Negative control; L: 100 bp DNA Ladder (Promega, United States). FPA (Fresh plant A), FPB (Fresh plant B), FPC (Fresh plant C) and FPD (Fresh plant D).

Furthermore, the current research also demonstrated successful amplification of the rbcL amplicon from the tea products (lanes P1–P4). The bands appeared bright and intact, approximately 600–700 bp, which aligns with the expected size range based on primer pairs used (approximately 500–600 bp). No contamination was

detected, as evidenced by the absence of bands in the negative control (Figures 4 and 5; lane −C, respectively).

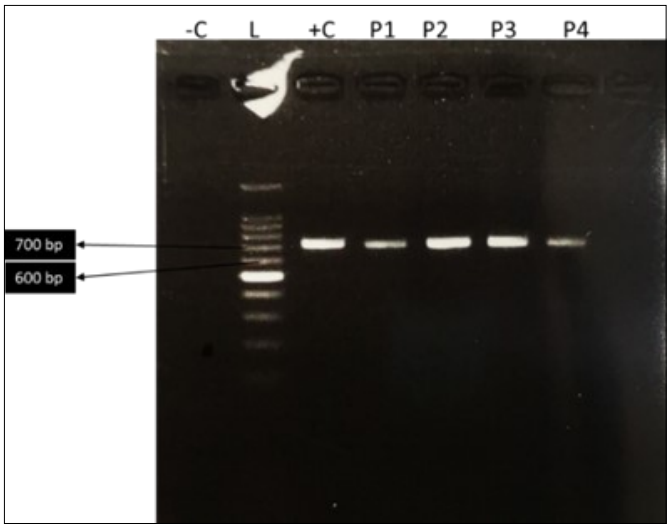


**Fig. 4.** PCR amplification for *rbc*L from tea herbal products run on 2% (w/v) agarose gel electrophoresis. +C: *rbc*L of *M. oleifera*; -C: Negative control; L: 100 bp DNA Ladder (Promega, United States). P1 (Product 1), P2 (Product 2), P3 (Product 3) and P4 (Product 4).

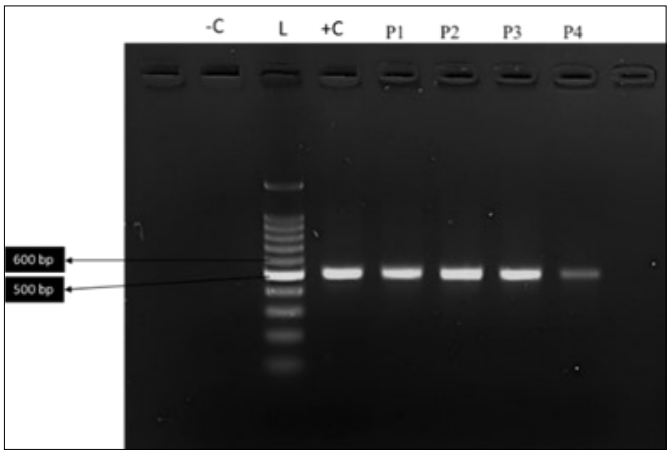


**Fig. 5.** PCR amplification for *ITS2* from tea herbal products run on 2% (w/v) agarose gel electrophoresis. +C: *ITS2* of *M. oleifera*; -C: Negative control; L: 100 bp DNA Ladder (Promega, United States). P1 (Product 1), P2 (Product 2), P3 (Product 3) and P4 (Product 4).

### *Sequence analysis of rbcL barcode for M. oleifera fresh leaves*

Several regions have been commonly used as plant barcodes, such as matK, rbcL, ITS, ITS2, and trnH-psbA (23). However, in this study, only two barcode regions, rbcL and ITS2, were specifically investigated and analyzed for their effectiveness in identifying the botanical origins of *Moringa* tea products. Nurhasanah and Nurmaya (24), have previously reported that rbcL is a suitable barcodes due to its universality, ease of amplification, analysis, and low level of mutation, compared to other barcodes. Additionally, rbcL is also considered the best-characterized plastid coding region in GenBank, offering sufficient discriminatory power (25).

As a part of the result analysis in the current study, BLASTn results of rbcL sequences from the fresh plant showed a high match and similarity (100%) to *M. oleifera* (accession number: MK165484.1) (Figure 6). The multiple sequence alignment (MSA) of rbcL sequences generated from the fresh plant sample revealed a highly conserved region with acceptable sequence variations among approximately 16 different species. Another multiple sequence alignment was conducted between sequences generated from the tea herbal product samples (P1–P4) and several sequences obtained from GenBank. Figure 7 demonstrated that all the herbal tea products (P1–P4) exhibited the highest sequence similarities with the rbcL sequence of *M. oleifera* from GenBank (MK165484.1) and the rbcL sequence obtained in the present study, confirming the presence of *M. oleifera* as the main ingredient in these herbal tea products.

From the multiple sequence analysis (MSA) results, as shown in Figure 8, the generated ITS2 sequence from this study, designated as FPD_ITS2, displayed the highest similarities with aligned sequences and only minimal variation compared to *M. oleifera* sequences from GenBank. As a comparison with the multiple sequence alignment among the herbal product sequences in Figure 9, the results concluded that all samples displayed high sequence similarities, with minimal variation, to the *M. oleifera* FPD_ITS2 and *M. oleifera* ITS2 sequences extracted from GenBank (accession no: MG548808.1).

### *Neighbour joining analysis*

The neighbour joining (NJ) analysis revealed that tea products P2, P3, and P4 were clustered together in the same clade as the *M. oleifera* rbcL sequence generated in this study, although with low bootstrap values. However, P1 formed an individual cluster close to the clade of *M. peregrina* (accession number: MF668600). Due to the low sequence variations between the species, the bootstrap values were not high in confidence as there were many shared branches among them. Based on this analysis, it can be summarized that the rbcL marker could only authenticate three tea products. This suggests that the rbcL is not efficient in identifying the raw materials in the tea products, especially P1. Since the packaging content of the P1 tea product was declared as *Moringa*, without specifying the species, there is a high possibility of containing other *Moringa* species, indicating substitution. As a result, even though rbcL can be amplified easily, it is not variable enough to distinguish across species. A previous study also indicated that rbcL supports the close association between various species of *Moringa* from different genotypes and regions (23).

The ITS2 barcode generated from the fresh leaves in this study showed the highest hit match (94%) to the *M. oleifera* sequences (accession numbers: MG548808.1 and MT827803.1) followed by 86% to another species, *M. ovalifolia* (accession number: JX092070.1) during BLASTn analysis. Based on the NJ analysis, the ITS2 from tea products had a monoclade branch (Figure 10). Although ITS2 was known to have a solid capacity to differentiate plants across species, in this case, the ITS2 DNA barcoding analysis alone may not be sufficient to resolve the species. A previous study also indicated that ITS2 could not discriminate *Moringa spp.* in selected herbal products from the Philippines (26). Another study revealed that the application of ITS2 for *M. oleifera* product was clustered with other congeneric species, including the

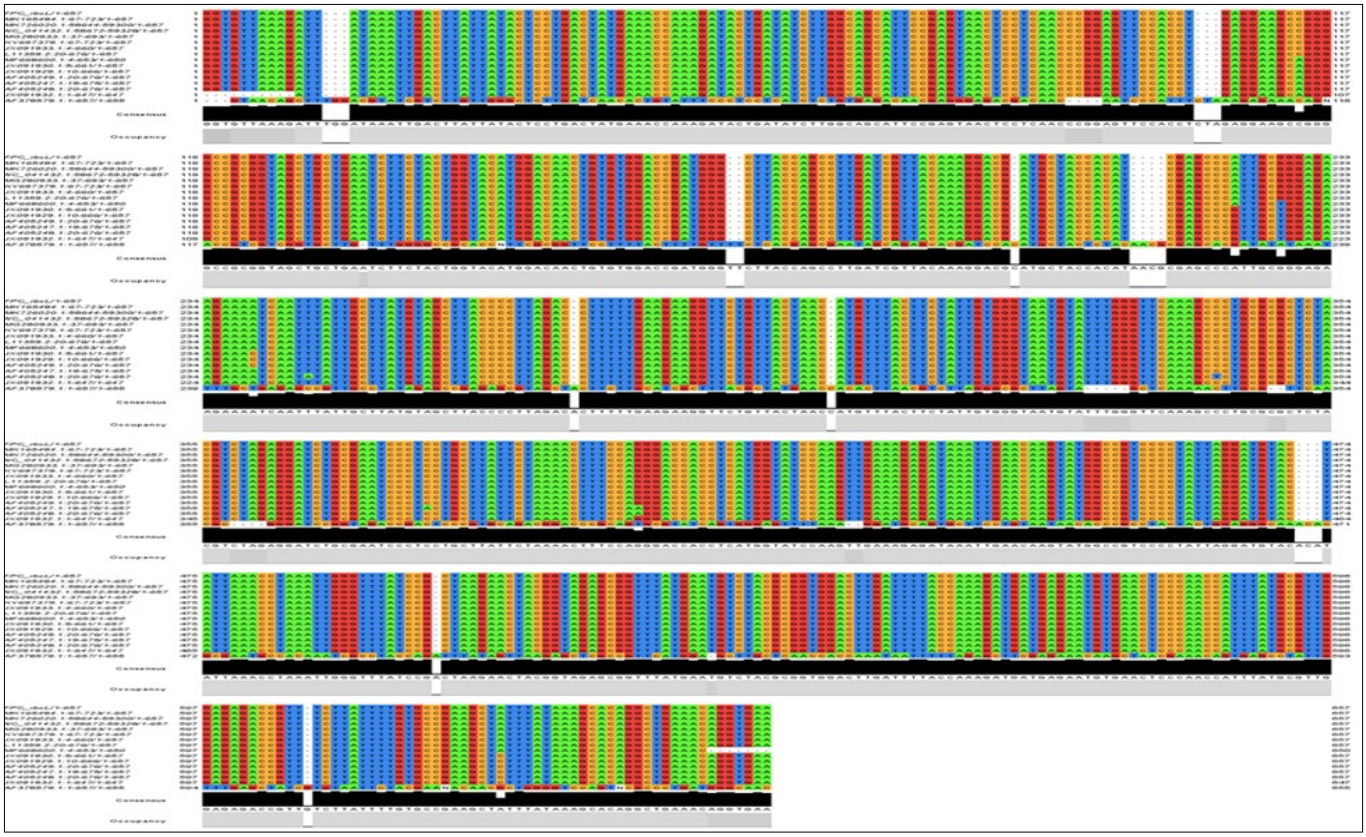

**Fig. 6.** Multiple sequences alignment (MSA) of *rbc*L gene from *M. oleifera*leaves with *rbc*L sequences retrieved from Genbank aligned using Jalview software.

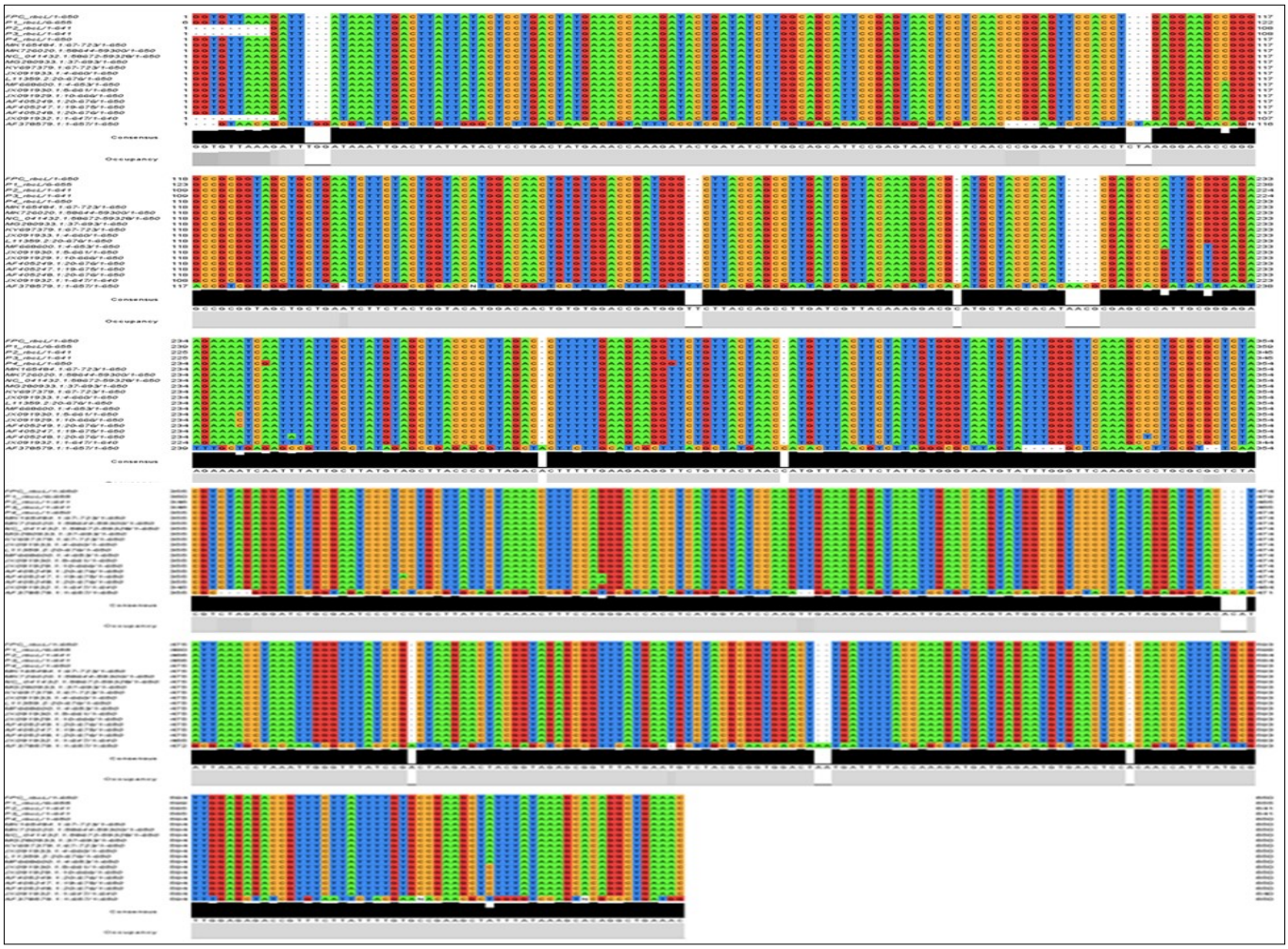

**Fig. 7.** Multiple sequences alignment of *rbc*L barcode regions from *M. oleifera* tea herbal products (P1 – P4) compared with *M. oleifera rbc*L reference barcode sequence (643 bp) established in this study (FPC_*rbc*L) and other sequences from GenBank.

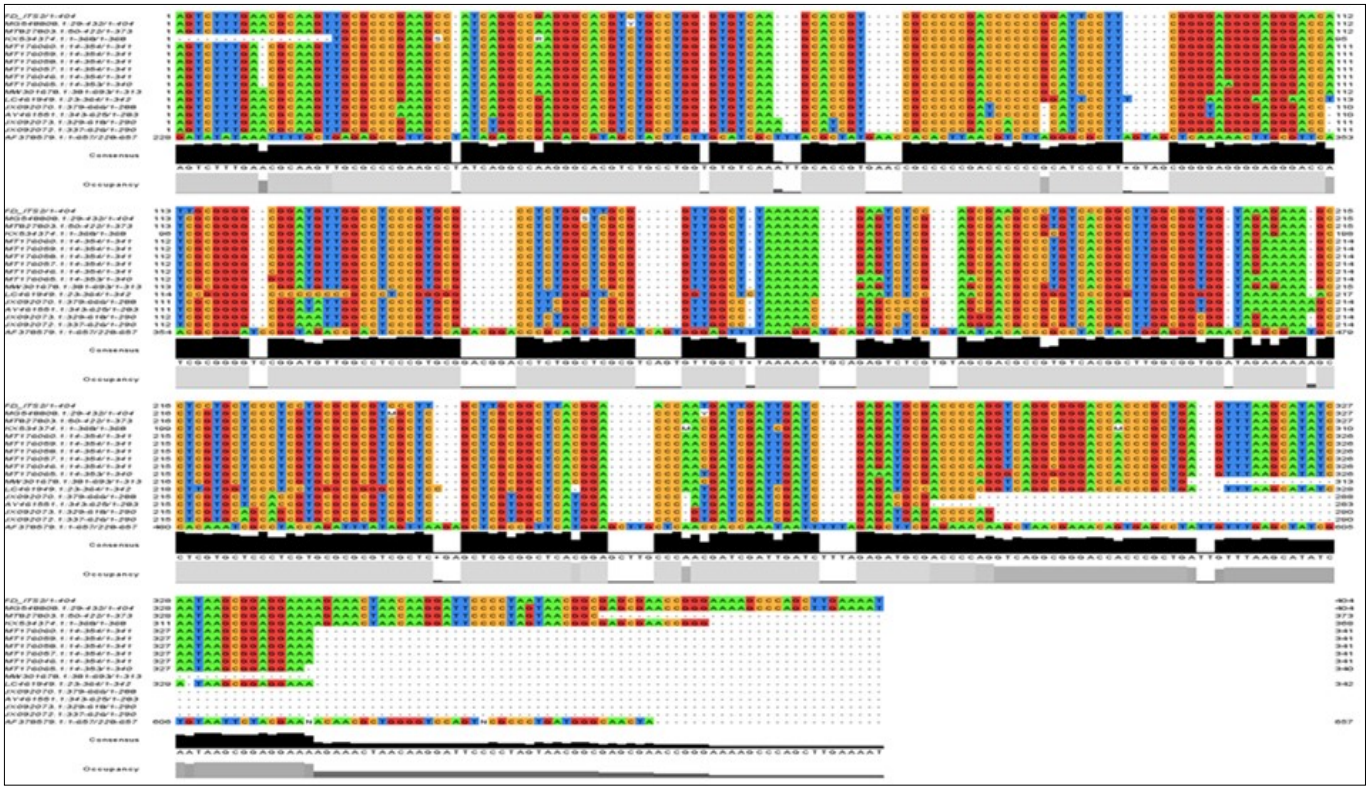

**Fig. 8.** Multiple sequence alignment of *ITS2* gene (404 bp) from *M. Oleifera* leaves (FPD_*ITS2*) with other *ITS2* sequences from GenBank were aligned using Jalview software.

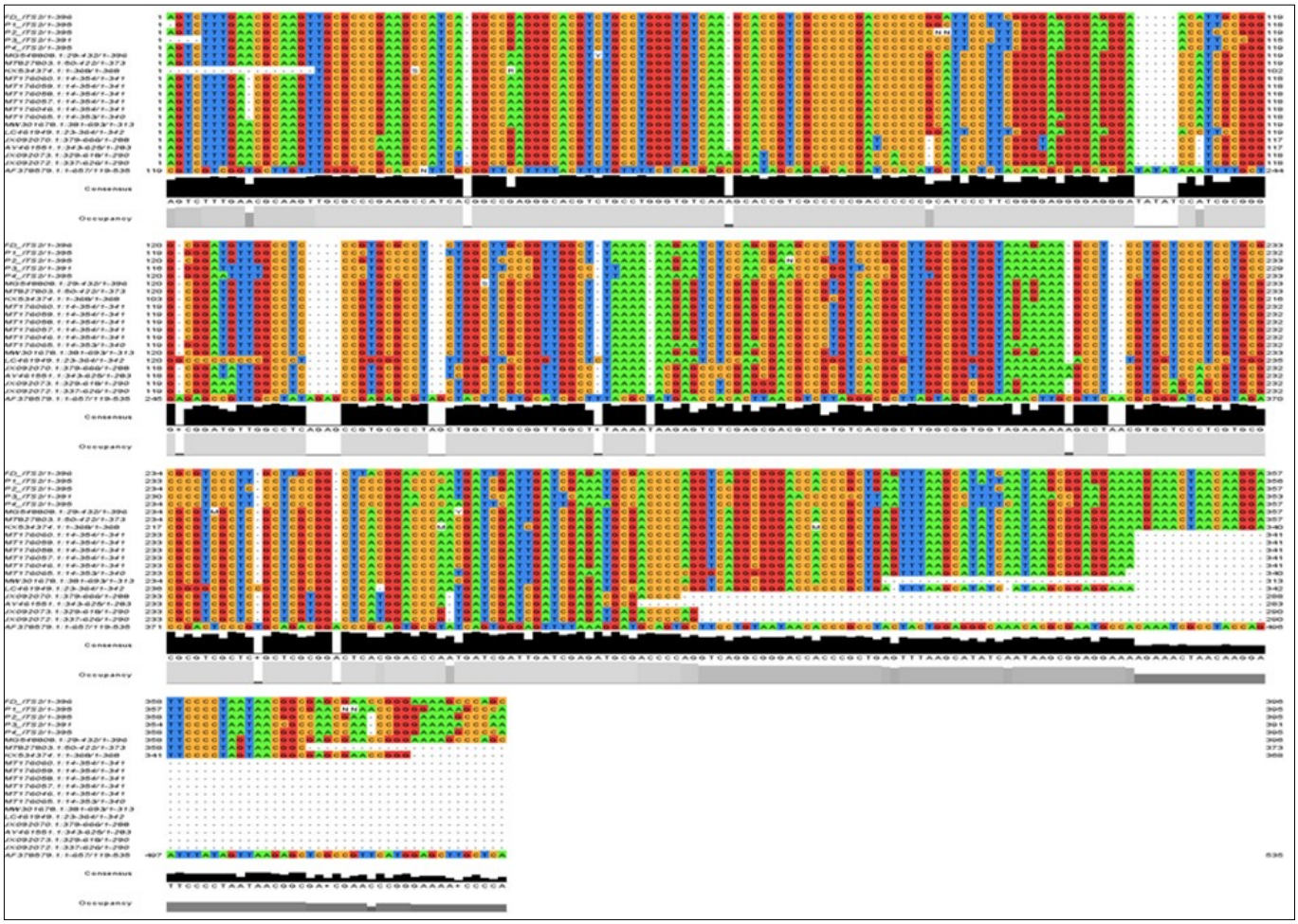

**Fig. 9.** Multiple sequence alignment of *ITS2* barcode regions from *M. oleifera* tea herbal products (P1 – P4) compared with *M. oleiferaITS2* reference barcode sequence established in this study (FPD_*ITS2*) and other sequences from GenBank.

expected species (27), suggesting that ITS2 lacks sufficient genetic variation to differentiate among closely related species. Therefore, ITS2 secondary structure analyses were performed to discriminate between *Moringa* species and tea products in this study.

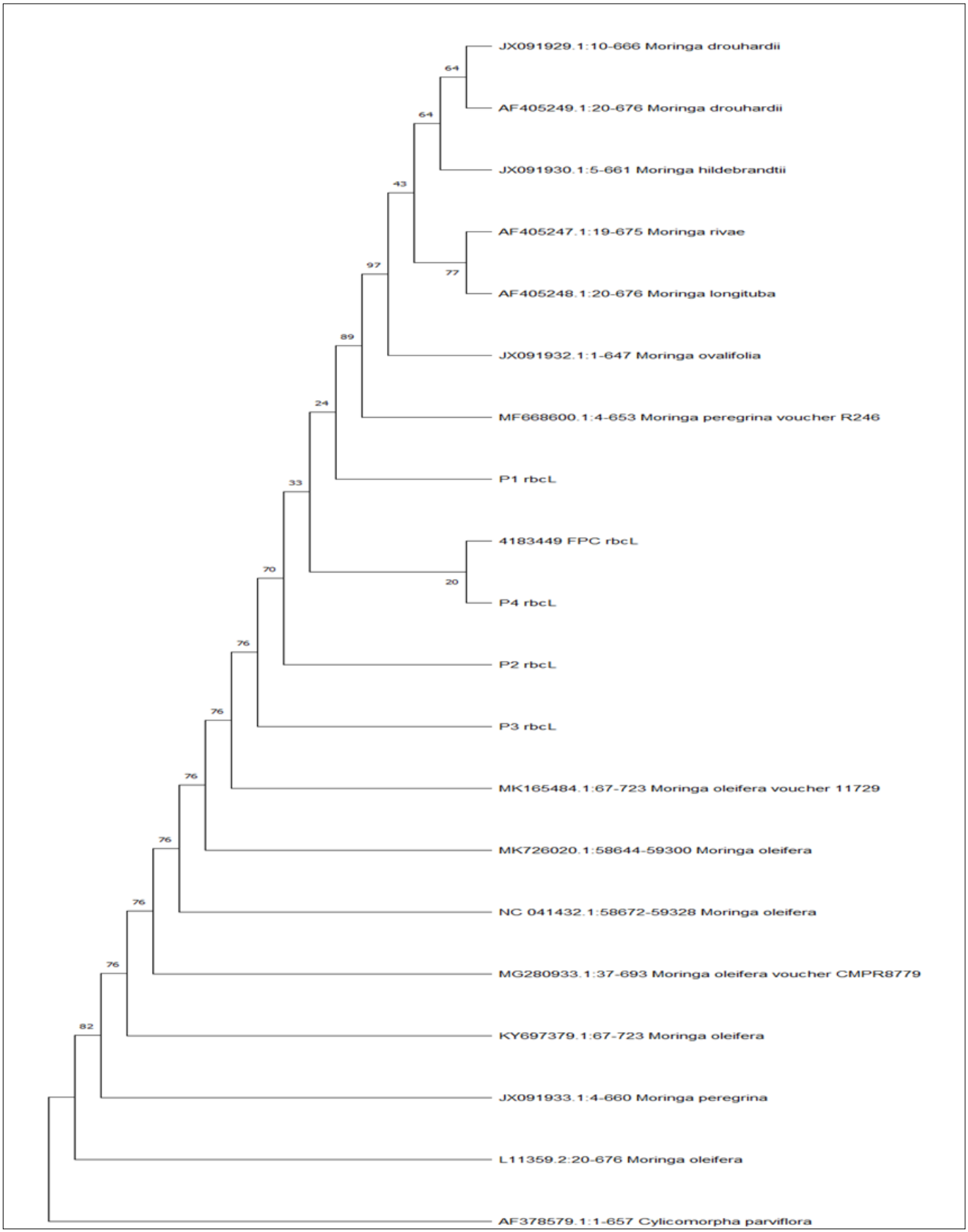


**Fig. 10.** The Neighbour-joining tree of *ITS2* from *M. oleifera* tea products (P1 – P4) compared to *ITS2* barcode sequences established in this study and other sequences retrieved from the GenBank.

### *Sequence analysis of the rbcL barcode for M. oleifera fresh leaves*

Secondary structure analysis revealed that ITS2 secondary structures of *M. oleifera* and its related species were different, as shown in Figure 11. Among these species, the secondary structures of ITS2 exhibited significant differences in the four helices, including the stem-loop number, size, position, and degree of the angles from the centre. The results demonstrated that the closely related Moringa

species could be differentiated based on their ITS2 secondary structures. The consensus secondary structure of *M. oleifera*, *M. ovalifolia*, and *M. rivae* had folded to form a central loop containing four helices radiating outward. The shortest helix IV was comparatively conservative in each structure of *Moringa* species, whereas the longest helix III showed higher variability. The secondary structures of *M. ovalifolia* and *M. rivae* included the highly conserved motif 5'-GATC-3' and differed from the structure of *M. oleifera* which consisted of 5'-GATT-3' at the apex of helix IV.

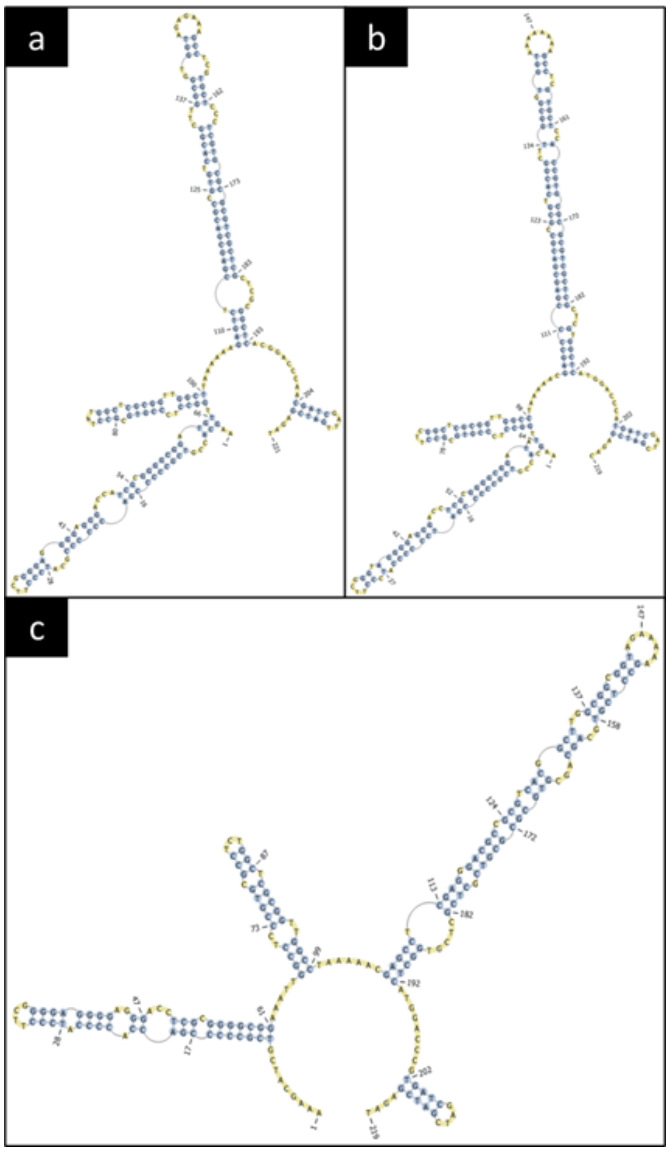


**Fig. 11**. The *ITS2* secondary structure of (**a**) *M. oleifera* plant, (**b**) *M. ovalifolia* and (**c**) *M. rivae*

The secondary ITS2 structures from the tea products (P1–P4) displayed a typical one-ring and four-helix structure. Each helix had stem-ring structures of different sizes and numbers, like *M. oleifera* (Figure 12). Previous reports highlighted that the ITS2 region and its secondary structure were conserved, which provided molecular and morphological characteristics that could be used for further improvements in species resolution (28), (9). The present study revealed that the ITS2 secondary structures from all the tea products had helices and motifs similar to those of the *M. oleifera* plant. The ITS2 secondary structure of *M. oleifera* revealed that the differences could be

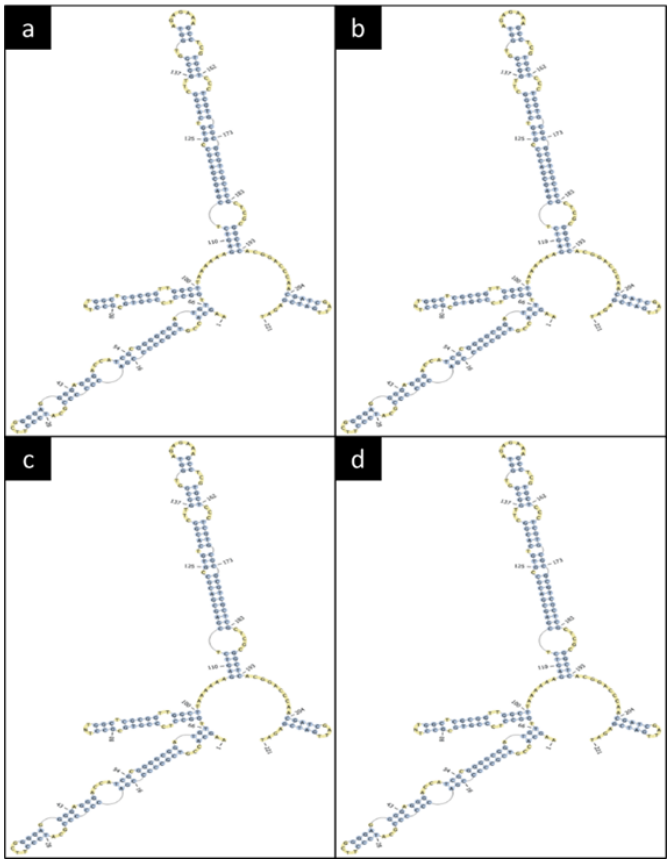


**Fig. 12.** The *ITS2* secondary structure of *M. Oleifera* herbal medicinal products (**a**) P1, (**b**) P2, (**c**) P3 and P4 (**d**)

identified in the stem length, loop number, and central ring compared to the closely related species (*M. ovalifolia* and *M. rivea*). All the secondary structures of herbal medicinal products consists of the same motif 5'-GATT-3' at the apex of helix IV, which are evident in the secondary structure of *M. oleifera*. Other *Moringa* species consisted of different motifs that did not match with the secondary structure of herbal medicinal products. Secondary structure analysis could evaluate the exact nucleotide base differences among the sequences to accurately delineate the species types and new lineages within the existing taxa (29). Therefore, this information can strengthen the recommendation that ITS2 DNA barcoding is a good marker for discriminating between *Moringa* species and its tea products. Thus, the findings from the present study strengthened the potential use of ITS2 secondary structure as a promising tool for identifying the authenticity of the Moringa-based products and can help to improve the discrimination between the closely related species.

## Conclusion

In conclusion, the standard reference DNA barcodes of rbcL and ITS2 for *M. oleifera* were efficiently produced to aid in the authentication of selected *M. oleifera* herbal medicinal products. The DNA barcode authentication of *M. oleifera* herbal medicinal products revealed that genomic DNA extraction was successfully performed, amplified, and sequenced for all four products (100%). While the DNA barcodes for *M. oleifera* herbal medicinal products were successfully amplified, the effectiveness of the rbcL and ITS2 barcodes in differentiating each product varied.

However, the secondary structure analysis demonstrated significant improvements in the authentication evaluation. The generated secondary structure successfully discriminate between the studied herbal medicinal products resulting in enhanced resolutions. Consequently, ITS2 was selected as a reliable DNA barcode for identifying the botanical origin of *Moringa* tea products.

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## Authors contributions

This study was designed, directed, and coordinated by Assoc Prof. Dr. Alina Wagiran as the principal investigator of the grant, provided conceptual and technical guidance for all aspect of the project and supervision. Nur Syazwani Mohd Nasarodin carried out the molecular genetic studies, sequence alignment and bioinformatics analysis as part of her thesis. The manuscript was written by Anisah Binti Akbar and commented on by all authors.

## Compliance with ethical standards

**Conflict of interest**: Authors do not have any conflict of interests to declare.

**Ethical issues**: None.

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