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Assessment of *matK, rbcL, rpoC1, trnA-trnH, ycf5,* and *ITS* for DNA barcoding of a *Mentha* species from Madinah city, Saudi Arabia

Rashid Ismael Hag Ibrahim*

Department of Biological Sciences, College of Science, King Faisal University, Al-Ahsa 31982, Saudi Arabia.

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ABSTRACT

Mentha (*Lamiaceae*) is a medicinal, aromatic plant known globally as mint. Madinah mint (Saudi Arabia) is grown and consumed as a drink, flavor for tea, spice for food, and a home remedy. In this report, *matK*, *rbcL rpoC1*, and *ycf5* genes, the intergenic region *trnA-trnH* from the plastid genome, along with the two nuclear loci internal transcribed spacers (*ITS1* and *ITS2*) were tested as DNA barcodes for Madinah mint. DNA was extracted, polymerase chain reaction was amplified using specific primers, and the products were sequenced. The partial sequences of the *matK* gene from Madinah mint are identical to those from *Mentha piperita*, while sequences of the *rbcL* gene were identical to *Mentha canadensis* and *Mentha spicata* discriminating this species from *M. piperita*. Phylogenetic analysis using *ITS1* or *ITS2* showed that Madinah mint is a basic taxon to *M. spicata* and *M. canadensis*. Phylogenetic analysis based on a combination of *ITS1* and *ITS2* sequences showed that Madinah mint is ancestral to other *Mentha* species. The same analysis using *rbcL* gene sequences again put Madinah mint as an ancestor of both *M. spicata* and *M. canadensis* as well as *Mentha suaveolens*, which might suggest that they have shared the same origin of the maternally inherited plastid genome.

1. INTRODUCTION

The *Lamiaceae* is a large family composed of 264 genera and around 7000 described species [1]. Square-like stems, whorl- or oppositely-positioned leaves, and irregular or zygomorphic flowers, usually with a 2-lipped corolla, characterize plants belonging to this family. Species of this family are mostly herbs or shrubs and rarely trees. They are endemic in two main centers of biodiversity: the Mediterranean basin, central Asia, and subtropical regions [2]. The members of this family are known as medicinal and aromatic herbs such as mint, sage, thyme, basil, rosemary, lavender, and oregano, which have been widely used as spices, teas, or traditional medicines [3-6]. In Saudi Arabia and the Arab peninsula, some of these species, including *Mentha* are commonly used as hot drinks or tea, home-based ailment relievers, or spices for cooking due to their contents of aromatic oils and other secondary metabolites [7]. Therefore, many species of the *Lamiaceae* family are considered commercial commodities, such as the Madinah mint in Saudi Arabia. Many species and cultivars of this family have been investigated for biochemical properties or taxonomic purposes [8-14]. While specific research on Madinah mint is limited, studies suggest that it possesses antioxidant, antimutagenic, and anticancer properties [15,16], and studies on related mint species suggest that it may possess antioxidant, antibacterial, antifungal, antiviral, and anticancer properties [17]. Further research is needed to fully understand the unique properties and potential health benefits of Madinah mint.

DNA barcoding is a technique developed by Hebert *et al*. [18] that relies on universal primers to amplify and sequence short universal DNA sequences. This technique has recently been applied as a universal tool for species authentication and identification [19-22]. The basic idea of this technique is to generate a standardized short DNA sequence(s) from any small tissue sample of a plant and compare it to any institutional library or international databases that contain reference sequences of the same or related species. This process will provide a rapid and reproducible taxonomic identification [18,23-29]. This method has been applied to identify members within the family *Lamiaceae* as well as to identify commercial processed spice species belonging to this family [10,11,30,31]. Herbs and spices are among trade commodities that can be adulterated, intentionally or accidentally, with morphologically similar plants. Hence, DNA barcoding would be a suitable technique to investigate and

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**Corresponding Author:*
Rashid Ismael Hag Ibrahim,
Department of Biological Sciences, College of Science,
King Faisal University, Al-Ahsa 31982, Saudi Arabia.
E-mail: ribrahim @ kfu.edu.sa

characterize these commercialized herbs possibly to the genus or species level [32-38].

DNA barcoding in animals is well established [39-41], but in plants, it is important to search for a suitable genomic region to perform DNA barcoding. However, some regions were suggested by scientists from around the Globe as well as the Plant Working Group of the Consortium for the Barcode of Life [42-44]. The suggested DNA regions include coding sequences from the plastid genome such as *matK*, *rbcL*, *rpoB*, *rpoC1,* and *ycf5*, genes as well as intergenic spacers such as *trnH-psbA*, *atpF-atpH,* and *psbK-psbI*. Moreover, the nuclear *ITS1* and *ITS2* have also been proposed as efficient plant DNA barcoding regions [45-51]. Recently, combinations of *matK* + *rbcL* or *ITS*s were proposed by the CBOL Plant Working Group (https://ibol.org) to increase the efficiency of plant species identification [43,52]. Even though a combination of the most appropriate regions for plant DNA barcoding and identification remains contentious and relies on trial and error [26,53-56].

The objective of this study is to investigate the applicability of DNA barcoding on the locally traded and available Madinah mint for authentication and identification. This study provided an evaluation of seven single candidate DNA barcoding loci and some of their combinations to identify the traded Madinah mint in Saudi Arabia.

2. MATERIALS AND METHODS

2.1. Plant Samples

Samples from traded Madinah mint were bought from the local market (Al-Ahsa, Saudi Arabia), which were used to test the performance of seven different candidate genomic regions for DNA barcoding analyses [Table 1].

2.2. DNA Isolation and Amplification

A sterile mortar and pestle were used to crush dry leaves (100 mg) under liquid nitrogen for DNA extraction. Dry leaves were used instead of fresh ones due to availability; they are more accessible in the Al-Ahsa region in eastern Saudi Arabia, whereas Madinah is located in the west. DNA was isolated using the Plant Genomic DNA Extraction Miniprep System (Viogene BioTek Corp., Taipei, Taiwan) to obtain high-quality DNA, free of polysaccharides or other metabolites that might interfere with DNA amplification. The protocol of the manufacturer was followed. Purified DNA concentration of samples was estimated both fluorometrically using a NanoDrop 2000c instrument (Thermo Scientific, DE, USA) and by comparison of ethidium bromide-stained band intensities with DNA standard (Edvotek, Washington, USA). The extracted DNA purity was above 1.83 at A260/A280.

2.3. Polymerase Chain Reaction (PCR) Amplification

PCR amplification for each candidate locus was performed using GoTaq® DNA Polymerase (Promega, CA, USA) in a 25 µL reaction volume according to the manufacturer's instructions. PCR protocols for the seven selected loci are listed in Table 1 that are all started with a denaturation step of 2 min at 95°C and ended with a final extension step at 72°C for 7 min.

One percent agarose gel size 7 × 7 cm was prepared for electrophoreses of amplified PCR products in ×1 TAE buffer. The gel was stained with ethidium bromide (0.5 µg/mL) in ×1 TAE buffer. Gel images were obtained using Benchtop 3UV™ transilluminator equipped with a BioDoc-It Imaging System (UVP, CA, USA). The size and presence or absence of amplified PCR products were determined on gel using a standard DNA ladder (Edvotek, Washington, USA). The ladder shortest fragment is 570 bp, and the longest is 23130 bp.

2.4. DNA Sequencing

PCR-amplified DNA was purified and bi-directionally sequenced by Macrogen Inc., Korea (http://www.macrogen.com). Forward and reverse sequences were obtained using the same primers that were used for PCR amplification, manually edited, and the 3' and 5' terminals were clipped to generate consensus sequences for each locus.

Table 1: Primers and PCR conditions for the seven selected DNA barcoding loci tested against Madinah mint in the present study [53].

Locus	Primer's Name	Primer sequences (5'-3')	Length (bp)	PCR cycle conditions	Cycles
matK	*matK*F	CgATCTATTCATTCAATATTTC	23	95°C 1 min 50°C 30 s 72°C 1 min	35
	*matK*R	TCTAgCACACgAAAgTCgAAgT	22		
rbcL	*rbcL*F	ATgTCACCACAAACAgAAAC	20	95°C 1 min 55°C 30 s 72°C 1 min	35
	*rbcL*R	TCgCATgTACCTgCAgTAgC	20		
trnA-H	*trnA-H*F	gTTATgCATgAACgTAATgCTC	22	95°C 1 min 55°C 30 s 72°C 1.5 min	35
	*trnA-H*R	CgCGCATggTggATTCACAATCC	23		
rpoC1	*rpo*CF	ggCAAAgAgggAAgATTTCg	20	95°C 1 min 53°C 40 s 72°C 40 s	40
	*rpo*CR	CCATAAgCATATCTTgAgTTgg	22		
ycf5	*ycf5*F	ACTTTAgAgCATATATTAACTC	22	95°C 1 min 53°C 40 s 72°C 40 s	40
	*ycf5*R	ACTTACgTgCATCATTAACCA	21		
ITS1	*ITS1*F	CCTTATCATTTAgAggAAggAg	22	95°C 1 min 50°C 30 s 72°C 1.5 min	35
	*ITS1*R	TCCTCCgCTTATTgATATgC	20		
ITS2	*ITS2*F	ATgCgATACTTggTgTgAAT	20	95°C 1 min 56°C 30 s 72°C 45 s	40
	*ITS2*R	gACgCTTCTCCAgACTACAAT	21		

PCR: Polymerase chain reaction

2.5. Data Analysis and Madinah Mint Delimitation

The Basic Local Alignment Search Tool (BLAST) [57] from the National Center of Biotechnology Information (NCBI) was used to search for relevant sequences in the NCBI and Barcode of Life Data databases. Clustal W [58] and Genetyx software (Genetyx, Tokyo, Japan) were used to align sequences obtained from the tested species and relevant sequences that were retrieved from the international databases. Pair-wise sequence comparisons of closely related plant species were conducted using BLAST2 Sequences [59] and Genetyx software (Genetyx, Tokyo, Japan). The Neighbor-Joining method of MEGA 11 [60] was used for phylogenetic analyses. The topologies of the phylogenetic trees were evaluated using the bootstrap re-sampling method of Felsenstein [61] and 1000 replicates. Phylogenetic trees have been built by applying the data to MEGA 11 [60], and the options were as follows; Statistical method: Neighbor-Joining, Test of phylogeny: Bootstrap method (1000 replicates), Model/Method: p-distance, Substitutions to Include: d: Transitions + Transversions, Rates among Sites: Uniform rates, Pattern among Lineages: Same (Homogeneous), Gaps/Missing Data Treatment: Pairwise deletion, and Select Codon Positions: 1st + 2nd + 3rd + non-coding.

3. RESULTS

3.1. Amplification and Sequencing of Seven DNA Barcoding Candidate Loci

A good yield of high-quality DNA was obtained from the Madinah mint samples under study. The first step of this work was to empirically test the universality of seven DNA barcoding candidate loci [Table 1]. Therefore, PCR amplification was conducted in different trials. The first trial was performed under standard PCR conditions starting from around 50 ng of template DNA. The second trial was applied only on loci that generated multiple and/or non-specific PCR products or did not generate any amplicons, where higher annealing temperature was applied. Loci and template DNA samples that failed to amplify were tried under lower stringency conditions through reduced annealing temperature and/or increased number of cycles. In case of failure of both trials, 0.5 or 1 µL of PCR products from both trials were then used as template DNA and re-amplified. PCR was considered a failure only in case of negative amplifications under all these different conditions. Samples of traded Madinah mint tested against the seven

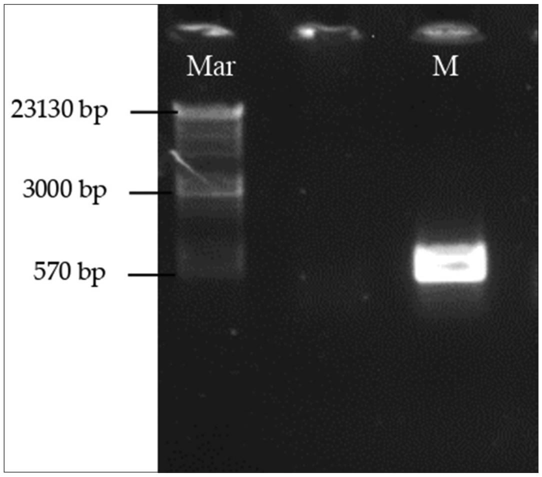

Figure 1: Polymerase chain reaction amplification of *ITS2* from Madinah mint (M). (Mar) refers to a DNA ladder (Edvotek, Washington, USA). The ladder shortest fragment is 570 bp and the longest is 23130 bp.

Table 2: Sequence identity, transitions, transversions, and indels of Madinah mint under study compared with other *Mentha* species based on sequences of *matK* or *rbcL* gene.

Species	Base	*M. canadensis*	*M. spicata*	*M. aquatica*	*M. longifolia*	*M. piperita*	*M. pulegium*	*M. suaveolens*	*M. cervina* and *M. arvensis*
					matK				
Madinah *Mentha*		99.7% (768 bp)	99.9% (768 bp)	99.9% (768 bp)	99.9% (753 bp)	100% (766 bp)	99.9% (768 bp)	99.9% (768 bp)	95.7 and 99.1% (768 bp)
	A			G (5)			T (21)		
	G	A (641)*	A (641)					A (641)	
	C				T (430)				
	T	C (595)							
									Indel (TTGGAA) (76–81)
					rbcL				
		100% (670 bp)	100% (666 bp)	99.7% (670 bp)	99.7% (670 bp)			99.9% (670 bp)	
	A			C (616)	G (311)				
	G								
	C				T (81)				
	T			G (625)				C (438)	

*All numbers between brackets are based on Madinah mint as a reference sequence

Medina Mentha	G C T T C G A A C A T A G G A G T T G C T A A G A G T C A A A T T C G T G G A A A A G A A C G G A T T G T
M. aquatica	G C T T C G A A C A T A G G A G T T G C T A A G A G T A A A A T T C G T G G A A A A G A A C G G A T T G T

Figure 2: Sequences alignment of *rpoC1* from Madinah mint and *M. aquatica* reveals a single single nucleotide polymorphism.

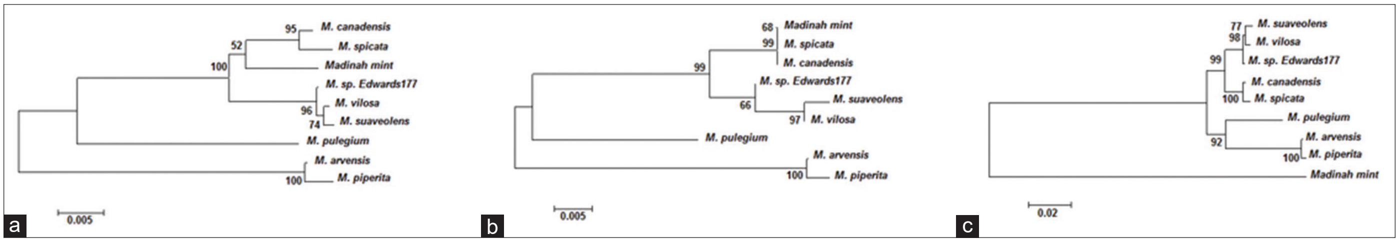

Figure 3: Evolutionary relationships of taxa inferred using the Neighbor-Joining method [87] based on (a) *matK*, (b) *rbcL,* and (c) *matK* + *rbcL* sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [61]. 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 p-distance method [88] and are in the units of the number of base differences per site. Codon positions included were 1st + 2nd + 3rd. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA 11 [60].

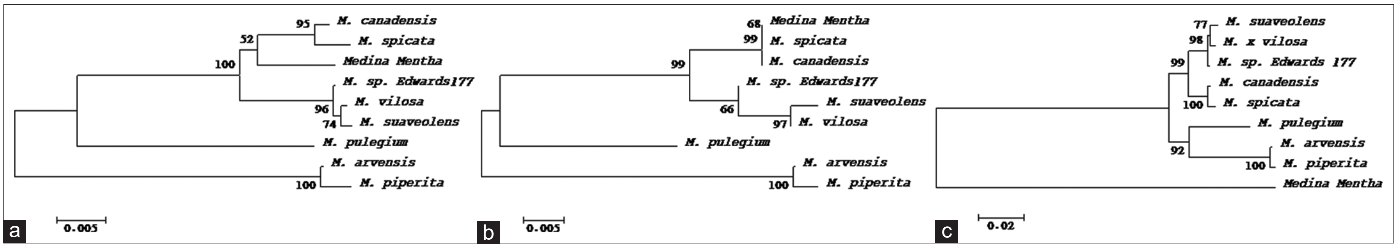

Figure 4: Evolutionary relationships of taxa inferred using the Neighbor-Joining method [87] based on (a) *ITS1*, (b) *ITS2,* and (c) *ITS1* + *ITS2* sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [61]. 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 p-distance method [88] and are in the units of the number of base differences per site. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA 11 [60].

selected loci exhibited 100% PCR amplification success after applying these different trials [Figure 1]. The amplified PCR products of the seven DNA loci were successfully sequenced, and high-quality bi-directional sequences were obtained. Sequences were submitted to the NCBI database, and accession numbers were acquired (*matK*; PV031553, *rbcL*; PV031554, *rpoC1*; PV031555, *ycf5*; PV031556, *ITS1*; PQ836415, *ITS2*; PQ836431).

3.2. Data Analysis

Based on a homology search of the database using sequences obtained from the plastid *matK* gene, Madinah mint is 100% (766bp) identical to *M. piperita*, but very similar to *M. spicata*, *Mentha aquatica*, *Mentha longifolia*, *Mentha pulegium* and *M. suaveolens* (99.9%; 753-68 bp), some transitions/transversions were observed [Table 2]. Using the *matK* gene sequence from Madinah mint as a reference, a transition from (G) to (A) at position 641 was observed in *M. canadensis*, *M. spicata,* and *M. suaveolens*, from (A) to (G) in *M. aquatica* at position (5), from (C) to (T) in *M. longifolia* at position 430, and from (T) to (C) in *M. canadensis* at position 595. One transversion from (A) to (T) was observed in *M. pulegium* at position 21. An indel (insertion/deletion) of 6 bp direct repeat was also observed in *Mentha cervina* and *Mentha arvensis* at the same position (76–81), which indicates that these later species share the same ancestral plastid genome that is different from other species, at least in this part of DNA.

When homology search of the NCBI database was performed using sequences obtained from the plastid *rbcL* gene of Madinah mint as a query, it was 100% (666-70 nt) identical to *M. canadensis* and *M. spicata* as well very closely related to *M. suaveolens* (99.9%; 670 bp) with only one base transition from (T) to (C) at position 438. However, it is also related to *M. longifolia* (99.7%; 670bp) with two transitions from (C) to (T) at position 81 and (A) to (G) at position 311. A base identity of 99.7% (670bp) was also detected between Madinah mint and *M. aquatica* with two transversions of (A) to (C) at position 615 and (T) to (G) at position 625 [Table 2].

When BLAST was applied to search the NCBI database using sequences obtained from the intergenic region *trnA-trnH* from Madinah mint as a query, it did not retrieve any *Mentha* spp. similarities except for *Chenopodium* spp. and other related taxa with base identity ranging between 80.8% in *Chenopodium foliosum* and 87.2% in *Chenopodium album*. Sequences of the *rpoC1* gene from Madinah mint retrieved a single similarity that is *M. aquatica* (99.7% base identity), where there is only one transversion from (C) to (A) at position 148 [Figure 2]. The *ycf5* sequences of Madinah mint resulted in similarities to *M. x piperita*, *M. spicata,* and *M. canadensis* (98.92%) [53].

Homology search of the database using sequences of *ITS1* distanced Madinah mint from *M. pulegium* and *M. piperita* (93.2% identity; 585-9 bp) as well as *M. arvensis* (93.4% identity; 580bp), but *M. suaveolens* scored 97.8% identity (584 bp). Yet, Madinah mint remained closely related to *M. spicata* and *M. canadensis* (98.1 and 98.5% identity, respectively; 584 bp). Likewise, a homology search of *ITS2* sequences showed that Madinah mint is distant from *M. pulegium* and *M. piperita* (92.4 and 92.9% identity, respectively; 302-50 bp) as well as *M. suaveolens* (96.8% identity; 312 bp), but remained closely related to *M. spicata* and *M. canadensis* (99.4% identity; 340-50 bp).

4. DISCUSSION

The BLAST, genetic distance and tree topology are some of the techniques applied to compare DNA sequences obtained from different living organisms for the purpose of molecular identification and delimitation [55,62-65]. DNA barcoding is a developed technique that uses specific DNA sequences to identify living organism species [18]. In plants, several DNA loci, either from the plastid organelle or nuclear genomes, were proposed by the Plant Working Group of the Consortium for the Barcode of Life [42-44]. These suggested DNA barcodes included coding sequences from *matK*, *rbcL, rpoB*, *rpoC1*, and *ycf5* genes, as well as *trnH-psbA*, *atpF-atpH,* and *psbK-psbI* intergenic spacers of the plastid genome. Besides, the nuclear *ITS1* and *ITS2* have also been proposed as efficient plant DNA barcoding loci [43,45,47-50,66]. Another suggestion from the CBOL Plant Working Group (www.barcoding.si. edu/plant_working_group. html) is to use combinations of *matK* + *rbcL* or *ITSs* for DNA barcoding so as to increase the efficiency of plant species identification [43,67,68]. Yet, a combination of two or more appropriate loci for plant DNA barcoding remains debatable and requires multiple trials [26,53,54,69-71].

In this study, partial coding sequences from *matK*, *rbcL*, *rpoC1*, and *ycf5* genes, beside noncoding sequences from the intergenic space *trnA-trnH* as well as *ITS1* and *ITS2* were tested against one of the most traded and famous *Mentha* in Saudi Arabia; Madinah mint. Sequences obtained from these loci of Madinah mint were applied to BLAST search on the international database.

The *matK* gene is often challenging to amplify and sequence using PCR due to its high sequence variability and complex secondary structure [44,72-75]. This study has successfully amplified and sequenced this region from the Madinah mint and was able to differentiate it from other *Mentha* species available on the database, except for *M. piperita*, which was 100% identical. The *matK* sequence of Madinah mint showed a single unique single nucleotide polymorphism (SNP) with *M. spicata*, *M. aquatica*, *M. longifolia*, *M. pulegium*, and *M. suaveolens* and 2 SNPs with *M. canadensis* [Table 2]. These SNPs could be very useful in the authentication and identification of *Mentha* species. SNPs were applied in authentication and identification of other plant species such as *Morinda umbellata* and *Matelea reticulata* [76], *Coffea canephora* and *Coffea congensis* [77], *Diospyros mespiliformis* and *Diospyros brandisiana* [28] and *Patrinia* species [78]. Phylogenetic analysis using the Neighbor-Joining method with partial sequences from *matK* gene was able to reveal the molecular evolution of Madinah mint that formed a basic phylogenetic clade and seems to be ancestral to most *Mentha* species that were retrieved from the database [Figure 3a].

In plants, the most commonly amplified and sequenced gene for taxonomic and phylogenetic studies is the plastid *rbcL* [79-81]. Hence, sequences of the *rbcL* gene from Madinah mint were obtained, and they were able to discriminate this species from other retrieved species, except that they were identical to sequences from *M. canadensis* and *M. spicata* [Table 2 and Figure 3b]. Plastid *rbcL* gene is well accepted in phylogenetic studies of plants, but delimiting a taxa to a single species based on *rbcL* gene alone is difficult, especially in closely related species [28,82-86] such as *Mentha*. Phylogenetic analysis using the Neighbor-Joining method and partial sequences from the *rbcL* gene revealed that Madinah mint is ancestral to *M. suaveolens*, *M. canadensis,* and *M. spicata*. Therefore, it is possible to say that the obtained partial sequences of *matK* and *rbcL* genes were fully capable of delimiting Madinah mint and can be applied to discriminate it from similar species due to these observed nucleotide differences.

The intergenic space *trnA-trnH* sequences from Madinah mint was not able to retrieve any similar *Mentha* species except for related taxa, probably due to a lack of similar sequences from *Mentha* species available in the database. Sequences of the plastid *rpoC1* gene from Madinah mint showed 100% base identity with *M. spicata*, but 99.78% with *M. aquatica* [Figure 2]. Yet, this partial *rpoC1* sequence can be applied as a DNA marker to differentiate between these two species, Madinah mint and *M. aquatica* based on detected SNPs. Alaklabi *et al*. [28] used SNPs found in a partial *rbcL* gene sequence to validate and differentiate *D. mespiliformis* tree. Sequences of the *ycf5* gene showed that Madinah mint is similar to *M. x piperita*, *M. spicata,* and *M. canadensis* (98.92%). The *ycf5* gene was applied in the study of inter/intra-specific variation of medicinal plant species [53].

In agreement with plastid loci above, the nuclear *ITS1* and *ITS2* were very successful and able to discriminate Madinah mint from all other *Mentha* species available on the database, despite the close relationships with some species. *ITS1* and *ITS2* can be applied as species-specific markers or DNA barcodes for the purpose of authentication and identification of this species of *Mentha* traded under the name Madinah mint in Saudi Arabia. The ITS region has been utilized as a DNA barcode to help identify over 21,000 plant species [51].

Phylogenetic analyses utilizing various genetic markers have provided insights into the evolutionary relationships of Madinah mint. The *matK* gene analysis positioned Madinah mint as a basal lineage to other *Mentha* species, excluding *M. cervina* [Figure 3a]. Conversely, *rbcL* sequence analysis placed Madinah mint as ancestral to *M. canadensis*, *M. spicata*, and *M. suaveolens*, while sharing a common origin with *M. aquatica* and *M. longifolia* [Figure 3b]. Combining both *matK* and *rbcL* data positioned Madinah mint as ancestral to all species except *M. longifolia* [Figure 3c]. When *ITS1* or *ITS2* sequences were used, Madinah mint appeared as ancestral to or a sister species of *M. canadensis* and *M. spicata* [Figure 4a and b]. However, the combined sequence analysis aligned with the results from *matK* and *rbcL*, positioning Madinah mint as a basal taxon to other species [Figures 3a-c and 4c]. This pattern is likely due to the shared origin of maternally inherited plastid genomes among these species [47].

5. CONCLUSION

Based on sequences alignment and phylogenetic analysis, this study demonstrated a very close relationship between Madinah mint and other related *Mentha* species based on at least partial coding sequences from two plastid genes (*matK* and *rbcL*) and two non-coding partial sequences from the nucleus (*ITS*s). The present findings showed that partial sequences of *matK* and *rbcL* genes were able to delimit the Madinah mint to its species level. The partial sequences obtained from the *trnA-trnH* intergenic space were not able to retrieve similar *Mentha* species probably due to lack of these sequences in the database. The sequences from *ycf5* gene differentiated Madinah mint from *M. x piperita*, *M. spicata* and *M. canadensis*. However, sequences from the nuclear *ITS1* and *ITS2* were able to position Madinah mint close to *M. spicata* and *M. canadensis*, yet, there were some differences. Therefore, it is recommended to use SNPs detected during this study in sequences of *matk* and *rbcL* genes and/or *ITS*s to authenticate and identify Madinah mint that is mixed in trade with other related species. Phylogenetic analysis using the Neighbor-Joining method and sequences from the *rbcL* gene were able to evolutionary position Madinah mint to a distinct phylogenetic clade with *M. suaveolens*, *M. spicata*, and *M. canadensis*, which may suggest that these three species have shared the same origin of the maternally inherited plastid genome.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

Most of the data generated and analyzed are included in this research article. If required, more data can be obtained from the corresponding author, Assistant Professor Rashid IH Ibrahim, via email at ribrahim@kfu.edu.sa.

12. PUBLISHER'S NOTE

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The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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