Original Article

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Molecular Identification and Phylogenetic Differentiation of Iranian Hypericum Species Using 18S rRNA Gene Barcoding and Bioinformatic Analysis

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Abstract

The *Hypericum* genus is renowned for its medicinal properties, yet its commercial products are often adulterated due to challenges in morphological identification. This study presents a molecular approach using 18S rRNA gene sequencing to identify and differentiate 12 *Hypericum* species collected from various regions of Iran. Genomic DNA was extracted, and the 18S rRNA ribosomal genes were amplified using specific primers, and sequenced. Besides, the morphologic characteristics of each sample were evaluated using light microscopy method. Bioinformatic analyses, including multiple sequence alignment, conserved domains shading and phylogenetic tree construction, revealed distinct clades corresponding to individual species, validating the efficiency of DNA barcoding of the studied *Hypericum* samples. This method offers a rapid, reliable, and reproducible tool for accurate species identification, which is essential for quality control in the herbal industry. The developed molecular identification methods can also be applied, with slight modifications, to identify other *Hypericum* specimens and similar plant species.

Keywords: 18S rRNA, DNA barcoding, Herbal authentication, Hypericum, Phylogenetic analysis.

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1. Introduction

Hypericum perforatum, commonly known as St. John's Wort (SJW), belongs to the family *Hypericaceae*, which was previously considered a subfamily of *Clusiaceae*. Also, in some sources, it is recognized as a part of the *Guttiferae* family (1).

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The relationships among Hypericum species have been elucidated through comprehensive analysis, drawing upon information gathered from numerous original studies covering morphology, distribution, floral vasculature, and, to a limited extent, cytology.

The *Hypericum* species encompasses a variety of forms, including trees, shrubs, and herbs. Due to its extensive spectrum, this genus exhibits diverse manifestations, such as wide-spreading roots or stems that spread

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but do not form roots from their nodes. Some species found in Africa even possess woody trunks (2).

Based on the constituents and metabolites derived from various species of *Hypericum*, including polycyclic poly prenylated acylphloroglucinols (PPAPs), xanthones, naphthodianthrones (e.g., hypericin and pseudohypericin), flavonoids, and flavonol glycosides (e.g., isoquercitrin and hyperoside), anthraquinones, aromatic compounds, benzophenones, bioflavonoids (e.g., amentoflavone), and phloroglucinol derivatives (e.g., hyperforin and adhyperforin), a wide range of pharmacological effects and biological activities are observed in these plants (3-5). These effects include the treatment of mild to moderate depression with antidepressant properties, as well as antiviral, antibacterial, anti-tumor, anti-inflammatory, and antioxidant properties (6, 7).

The concentration and sometimes the specific types of ingredients vary among different genera. For instance, *H. sampsonii* is a rich source of PPAPs and shows potential as a PDE4 inhibitor for treating gastrointestinal and traumatic injuries (8, 9). *H. foliosum* is used traditionally as a diuretic, hepatoprotective, and antihypertensive agent (10). Extracts of *H. perforatum* are utilized for psychiatric issues like depression, obsessive-compulsive disorder, attention-deficit hyperactivity disorder, menopausal symptoms, somatic symptom disorder, male sexual and reproductive health (SRH), paranoia and bed wetting, vertigo, hyperhidrosis, melancholy, and skin conditions such as wounds and muscle pain, burns, puncture wounds. In China, it is also used for enteritis, mastitis, dysentery, scrofula, and contusion problems (8, 11-13).

Hypericum species grow in a variety of climates. Optimal growing conditions include well-drained, coarse-textured soils with a slightly acidic to neutral pH. They can thrive in both sunny and partially shaded environments. They are typically found in diverse habitats in temperate regions and can also be observed in high mountains of tropical regions and areas with extreme dryness, high temperatures, and high salinity. These species are believed to be native to temperate and subtropical regions of North America, Europe, Asia Minor, Russia, India, and China; however, they have been propagated and can now be found in many parts of the world. Its distribution extends to North and South America, Australia, New Zealand, and other regions (14).

This species is widely distributed across Western Asia, Central Europe, Northern Africa, Indonesia, North and South America, and Australia. In Iran, it can be found in Gorgan (including Gonbad-e Kavus and Golestan Forest), Mazandaran (Haraz Valley, Karehsang, Talar Valley, Pol Sefid, Chalus Valley, Pol Zanguleh, Kalardasht), Gilan (Lahijan, Rasht to Astara road), Azerbaijan (northeast of Ardabil, Tabriz, Urmia, Karabakh, Ali Balagh forest, Sablan mountain), Kurdistan (Sanandaj, Mahabad), Hamedan (Abbas Abad, Hamedan, Koh Alvand, Nahavand, Borujerd), Lorestan (Bisheh Koh Sawers), Fars (Tal Khosravi), Khorasan (Bojnord, Tape Maraveh, Mashhad), Tehran (Pas-e-Qaleh, DaliChay, Haft Hoz Valley), Qazvin, Karaj, Damghan, and Semnan (15).

The DNA barcoding method has several advantages over older methods to find the possible herbal samples adulteration (Figure 1).

In this study, we collected twelve samples from different regions of Iran. Following the extraction of their genomic DNA, the 18S rRNA sequences were targeted and amplified. The replicated ribosomal genes were compared with each other and with similar genes from other *Hypericum* species, leading to the identification of multiple sequence alignments and the construction of a phylogenetic tree. The conserved domains will be identified and used to distinguish potential adulterations in *Hypericum* samples. This is the first detailed phylogenetic and molecular authentication of 12 Hypericum samples from Iran based on the 18S rRNA gene. The integration of microscopic, molecular, and geographic data for a comprehensive authentication pipeline will provide valuable data for application in real-world herbal market authentication to detect adulteration.

2. Materials and Methods

2.1. Herbal Sample Collection

Comparing the advantages and disadvantages of old methods and DNA barcoding	Traditional methods	DNA Barcoding
Time consuming	✓	✗
Ability to use in large scale	✗	✓
Limitations on types of samples	✓	✗
Industrial scale	✗	✓
Show phylogenetic relationships	✗	✓
Show evolutionary history	✗	✓
Need high skills	✓	✗
Advanced equipments	✓	✓
Skilled operators	✓	✗
Need particular part of plant	✓	✗
Cost effectiveness	✗	✓
User friendly	✗	✓
Simplicity	It depends on method	✓
Speed	✗	✓
Accuracy	It depends on method	✓

Figure 1. Comparing the advantages and disadvantages of old methods and DNA barcoding.

The herbal samples were purchased from various herbal shops located in different regions of Iran. Each sample was identified based on its organoleptic characteristics and confirmed through DNA sequencing. In Table 1 and Figure 2, the scientific name, type of species, and geographical location of each sample used are presented. *Hypericum* spp. coded from PM 1067 to PM 1078, provided in Table 1.

2.2. Microscopic Analysis

Figure 2. Location of Hypericum Species in Iran.

Table 1. Classification of different species of Iranian Hypericum.

Sample number	identification number	species name	Place of sample collection	Geographical coordinates
S1	PM 1071	Hypericum scabrum	A sample of herbal medicine store in Ahvaz	31.3183° N 48.6706° E
S2	PM 1076	Hypericum elongatum	A sample of herbal medicine store in Bandar Abbas	27.1963° N 56.2884° E
S3	PM 1075	Hypericum elongatum	A sample of herbal medicine store in Tehran	35.7219° N 51.3347° E
S4	PM 1078	Hypericum perforatum	A sample of herbal medicine store in Kerman	30.2839° N 57.0834° E
S5	PM 1073	Hypericum scabrum	A sample of herbal medicine store in Yazd	31.8974° N 54.3569° E
S6	PM 1077	Hypericum helianthemoides	A sample of herbal medicine store in Sanandaj	35.3119° N 46.9964° E
S7	PM 1070	Hypericum scabrum	A sample of herbal medicine store in Yasuj	30.66722°N 51.57972°E
S8	PM 1072	Hypericum scabrum	A sample of herbal medicine store in Babol	36.5387° N 52.6765° E
S9	PM 1074	Hypericum scabrum	A sample of herbal medicine store in Mashhad	36.2972° N 59.6067° E
S10	PM 1067	Hypericum elongatum	A sample herbal medicine store located in Shiraz (Chehel Giah) =[CG]	29.5926° N 52.5836° E
S11	PM 1068	Hypericum elongatum	A sample of an herbal medicine store located in Shiraz (Darvaze Kazeron) =[DK]	29.5926° N 52.5836° E
S12	PM 1069	Hypericum perforatum	A sample herbal medicine store located in Shiraz (Adlu Zerehi) =[AZ]	29.5926° N 52.5836° E

At this stage, various parts of *Hypericum*, including flowers, leaves, stems, and aerial organs of the plant specimen, were obtained, and all parts were powdered. Subsequently, microscopic characterization was performed. The process involved placing each sample under a microscope and taking photographs. This procedure plays a significant role in identifying the macro and microscopic features of plants and their properties. Initially, the dried parts of the plant were ground into powder using a porcelain mortar. The powders were sieved through a 70-mesh sieve, and 5 gr of the sample was accurately weighed using a balance with a precision of 0.01 gr. The sample was transferred to a test tube, and 5 mL of 60% chloral hydrate was added; then, it was heated over a flame. After boiling, it was centrifuged, and the supernatant layer was decanted. Then, 50 mL of distilled water was added to the residue, and the mixture was centrifuged again. The remaining plant residues in the test tube were poured into a watch glass and a few drops of phloroglucinol-chloric acid. Then, a slide was prepared from the sample, and each part and aerial organ was examined under a light microscope. Photographs of each sample were taken using a digital camera.

2.3. Genomic DNA Extraction

The DNA extraction process from Hypericum species involved several steps. Initially, 200 mg of lyophilized Hypericum was finely ground. Subsequently, 400 µL of extraction buffer was added to the ground material and thoroughly mixed. The samples were cen-

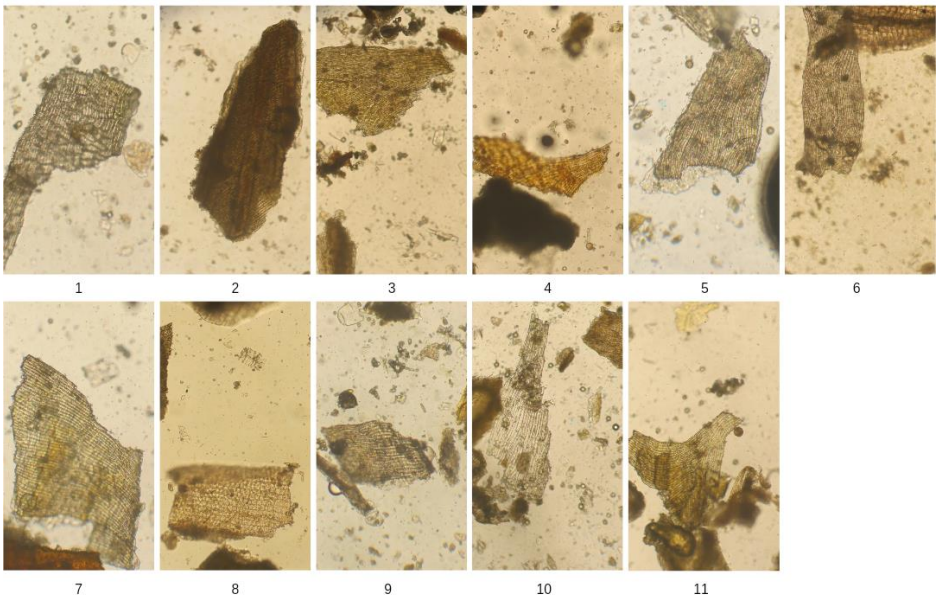

Figure 3. Fruit endocarp tissue fibers from the studied Hypericum samples.

trifuged at 7500 g for 10 minutes, after which genomic DNA was extracted from the biomass using the DNP™ Kit (high-yield DNA purification kit) according to the manufacturer's protocol. Next, five µL of protease solution was added to the obtained mixture, and the resulting mixture became completely uniform. It was then placed in a heat block for 30 min at a temperature of 55 °C. Next, 400 µL of lysing solution was added, and the mixture was vortexed for 15 seconds.

At the end of this step, a clear solution remained without any visible particles or sediment. Adding 300 µL of precipitating solution and vortexing the samples for 5 seconds helped to precipitate the DNA in the samples. Then, the samples were centrifuged at 12000 g for 10 min. The supernatant liquid was slowly separated, and to ensure it was completely drained, the microtubes were placed upside

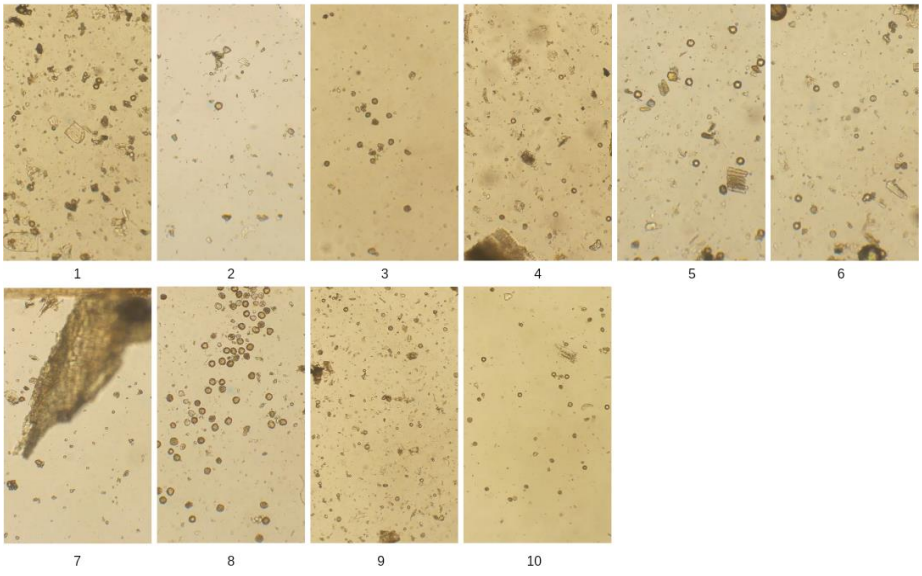

Figure 4. Pollen grains from the studied Hypericum samples.

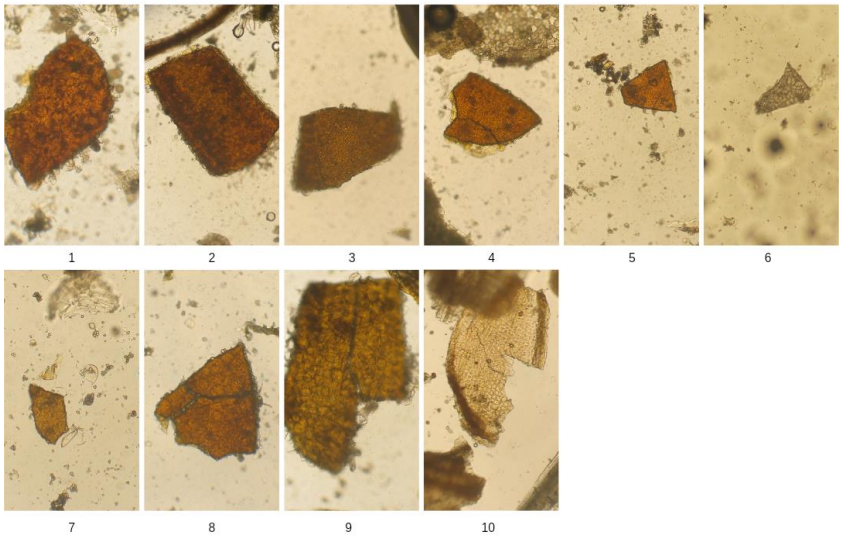

Figure 5. Seed coat texture from the studied Hypericum samples.

down on the filter paper for a few seconds, allowing the remaining liquid to leak into the filter paper. Then, 1 mL of washing buffer was added to each microtube, and the samples were vortexed for 5 seconds. Like the previous step, the samples were centrifuged at an acceleration of 12000 g for 5 minutes. Here, too, the supernatant was separated, and again, the microtubes were placed upside down on the filter paper for a few seconds, allowing the remaining liquid to leak into the filter paper.

Additionally, the microtubes were placed in a heat block at 65°C until the remaining liquid in them had evaporated entirely. Finally, to purify the DNA in the pellets, a volume of 50 µL of the DNA-dissolving buffer was slowly and gently poured onto the inner wall of the microtube with a circular motion. The samples were incubated at 65 °C for 5 minutes. Ultimately, the samples were centrifuged at 12000 g for 30 seconds. After centrifugation, the supernatant liquid was discarded, and the pellet was washed with 1 milliliter of 70% ethanol. Upon removal of the ethanol, the pellet was dried entirely. Subsequently, 30-50 µL of distilled water was added to the dried pellet, and the mixture was gently mixed; the resulting supernatant contained purified DNA.

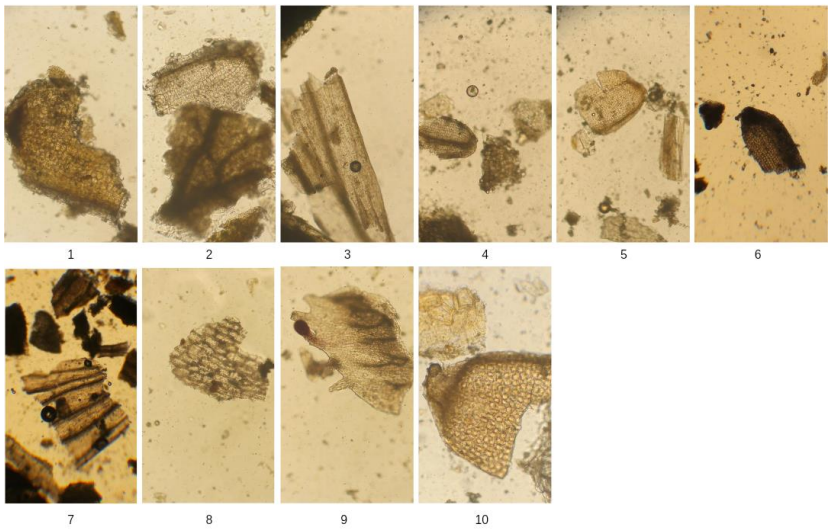

Figure 6. Petal texture from the studied Hypericum samples.

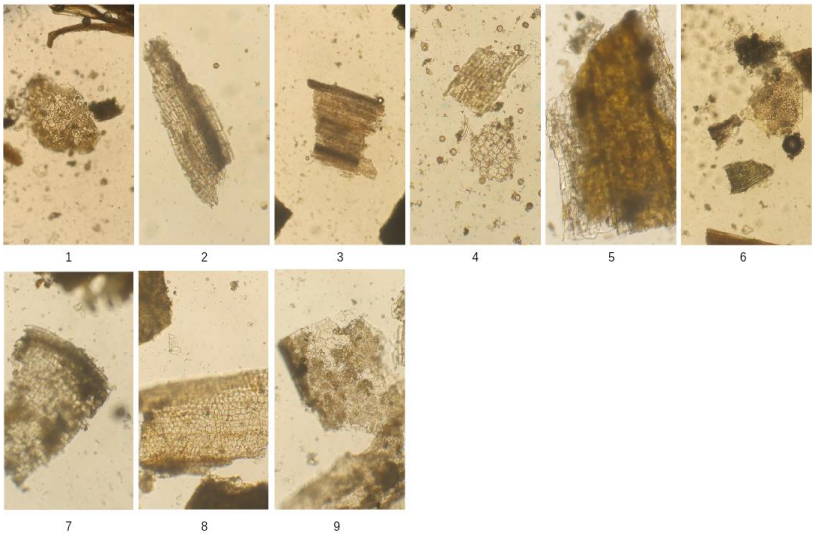

Figure 7. Stem parenchyma tissue from the studied Hypericum samples.

Purified DNA was kept at -20 °C. The resulting supernatant liquid serves as the template for PCR (16).

2.4. PCR Protocol

PCR was conducted to amplify the 18S rRNA gene from the selected plants utilizing gene-specific primers and master mix, following the manufacturer's instructions.

After mixing the materials, including forward primer, reverse primer, master mix, DNA template, and sterile deionized water, the microtubes were vortexed and then placed in a microcentrifuge. The microtubes were then placed in the PCR thermocycler. The PCR protocol included an initial denaturation step, named pre-denaturation, at 95 °C for 5 min, followed by 30 cycles of the following steps: denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, and elongation at 72 °C for 90 seconds. This was followed by a final cycle, named extension, at 72 °C for 5 min. PCR was performed using specific primers 5´-GTCAGAGGTGAAATTCTTGGATTTA-3´ and 5´-AGGGCAGGGACGTAATCAACG-3´ as the reverse primer. Taq polymerase was introduced to the reaction after the initial denaturation step (16-19). The employed primers amplify an expected product size of 400-700 base pairs.

2.5. DNA Electrophoresis

DNA sequences were separated using agarose gel electrophoresis to resolve their molecular weights. To distinguish species with similar molecular weights but different net charges, an electrophoresis system was employed, involving isoelectric focusing on an immobilized pH gradient. Samples were sub-

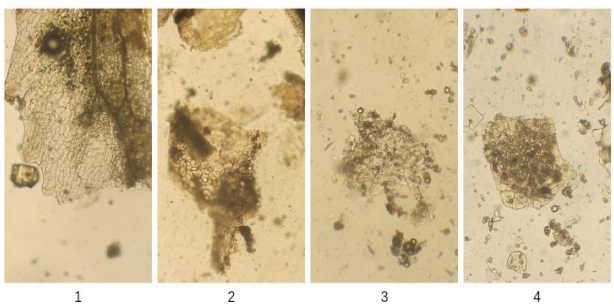

Figure 8. Epidermis of the leaf tissue from the studied Hypericum samples.

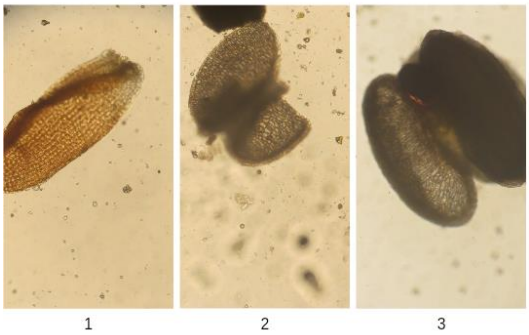

Figure 9. Another tissue from the studied Hypericum samples.

jected to electrophoresis in a 1% (w/v) agarose gel using TBE buffer supplemented with 1 µg/mL ethidium bromide.

Next, the gel cassette was placed in the electrophoresis tank, and X0.5 TBE buffer was poured into the electrophoresis tank (until the wells were filled with the buffer and the gel was entirely covered by the buffer). 100 bp ladder was used to check and estimate the size of DNA fragments in the samples (final PCR product). After this step, the samples were injected into the wells, and the electrophoresis process was performed with a voltage of 50 millivolts for 80 min. A single 700 bp DNA fragment was excised and extracted from the gel using the Core Bio Gel Extraction Kit. Sequence similarity searches of the 18S rRNA gene from Hypericum and its putative protein were performed using BLAST via the NCBI and CLC Sequence Viewer software version 8 (17). The Mega software (Version 11) was employed for phylogenetic analysis (20). The Neighbor-Joining algorithm, with 500 replicates as bootstraps, was employed to construct the phylogenetic tree.

3. Results and Discussion

3.1. Microscopic Analysis

In the process of microscopic analysis, the first step involves analyzing the fingerprint and conducting a morphological analysis of the plant sample being studied. Different components of the Hypericum plant, including the flower, leaf, and stem (an aerial organ), were prepared. All the components were then powdered, and the process of pulverization was completed. The process of detailing the parts under the microscope was completed, and detailed photographs of the organs were prepared.

Fruit endocarp tissue fibers in different samples collected are shown in Figure 3. Pollen grains in different samples collected are depicted in Figure 4. The seed coat textures of different collected samples are presented in Figure 5. Petal texture in different samples collected in Figure 6. Stem parenchyma tissue in different samples collected in Figure 7. A

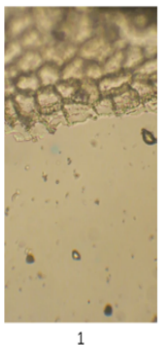

Figure 10. Fruit exocarp tissue from the studied Hypericum samples.

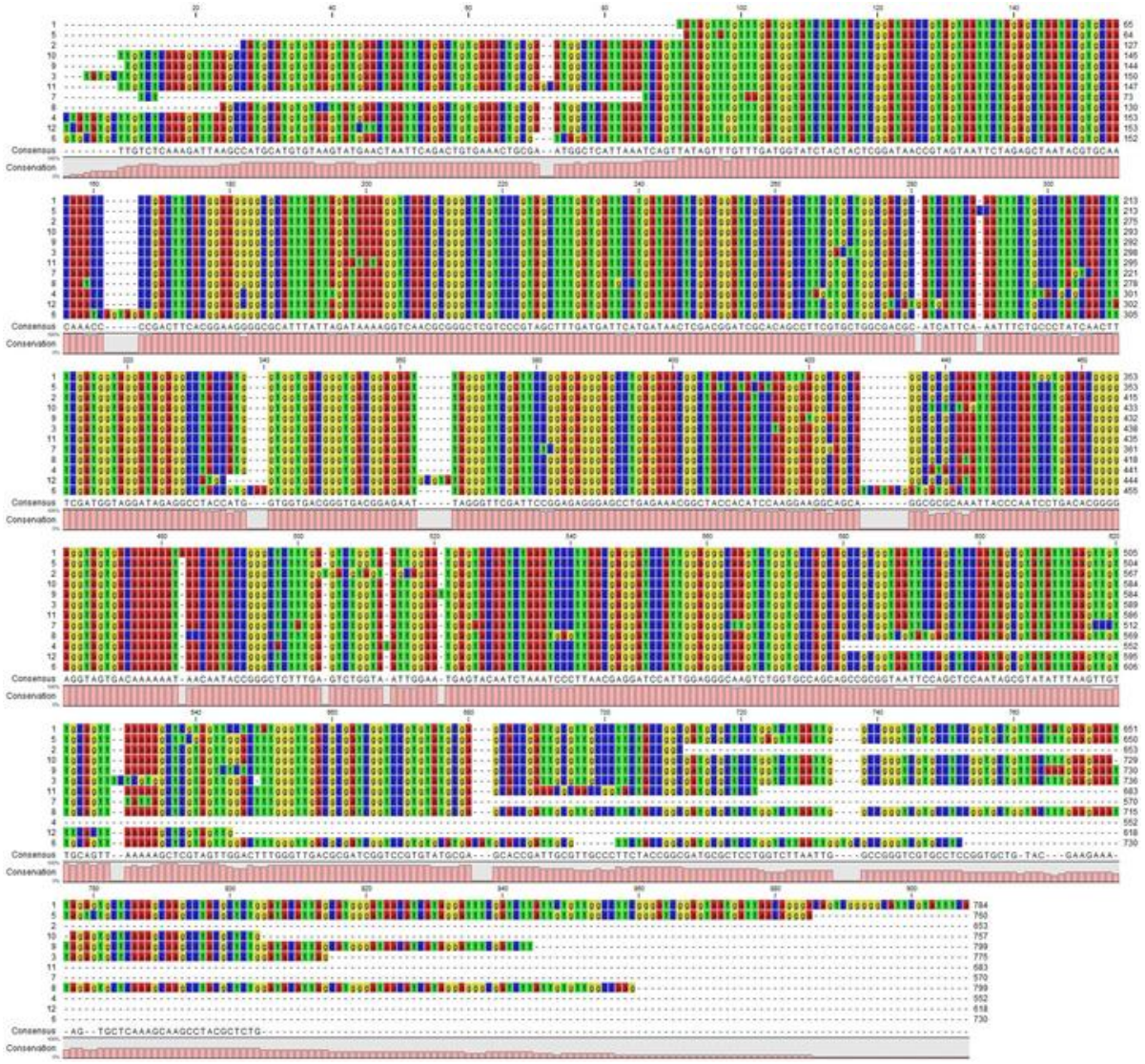

Figure 11. The similarity between the amplified 18S rRNA genes from different Hypericum samples, the conserved domains (%) are shown by pink color. The name of each studied sample is presented in Table 1.

part of the epidermis of the leaf tissue in the different samples collected in Figure 8. Part of the anther tissue in the different samples collected in Figure 9. Also, the fruit exocarp tissue in one sample is shown in Figure 10.

3.2. DNA Barcoding Method

This approach involves sequencing standardized DNA sequences derived from a specific genomic region as a tool for species identification (21). This method, which utilizes DNA sequencing to identify the order of guanine, adenine, thymine, and cytosine, enables the easy identification of plant species by detecting common gene sequences (22). It is a reliable, rapid, and relatively straightforward approach. While various methods exist today for examining the genome and identifying plants based on gene sequences and nucleotide structures, including techniques rooted in DNA and RNA, as well as detection through metabolites, transcription methods, proteins, and comprehensive phenotypic analyses, the emphasis here lies on identification methods utilizing DNA and unique gene sequences present in different plants. This includes the following components: DNA sequencing, genetic profile, structural and functional genom-

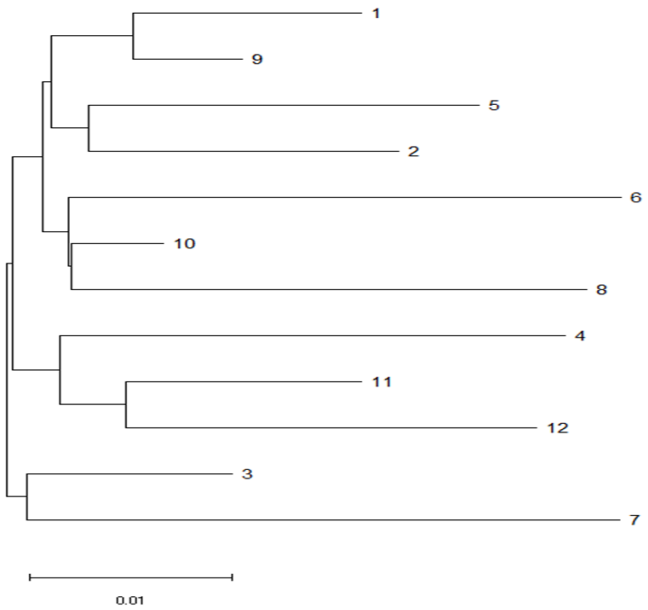

Figure 12. Phylogenetic tree showing the phylogenetic relationship between 12 studied Hypericum samples, constructed using the Neighbor-Joining method on 18S rRNA sequence data in MEGA software version 11. The name of each studied sample is presented in Table 1.

ics, and genetic mapping (23).

3.3. Importance of Ribosomal Genes

Because evolution progresses slowly in the nuclear 18S rRNA gene in eukaryotes, these genes are ideal choices for distinguishing between different samples. In the classification of different species with nuclear ribosomal RNA genes, similarity-based or BLAST-based methods are used (24). Results of many experiments indicate that ribosomal genes are promising candidates for individualizing the phylogeny and biodiversity of plant species. By utilizing bioinformatics knowledge and data and arranging them with a computer, or more specifically, in silico bioinformatics methods, biological patterns and relationships between different generations emerge. This type of information significantly aids scientists in discovering gene pathways and family relationships among various plants (25).

3.4. CLC Sequence Analysis

CLC Sequence Analysis is a bioinformatics tool specifically developed for the analysis, visualization, and interpretation of biological sequences, including DNA, RNA, and protein sequences. This software suite is extensively utilized in genomics, transcriptomics, and proteomics to enhance the understanding of complex biological data. CLC offers a variety of alignment algorithms, enabling users to compare sequences and identify conserved regions, which is essential for evolutionary studies and functional annotation. Users can also construct phylogenetic trees based on sequence data, aiding in the exploration of evolutionary relationships among species or genes. In Figure 11, each nucleotide is represented by a distinct color: adenine (A) is shown in red, thymine (T) in green, cytosine (C) in blue, and guanine (G) in yellow. Additionally, beneath each domain, the percentage of similarity of specific nucleotides across different species is highlighted in pink (26).

3.5. Phylogenetic Trees

Phylogenetic trees are graphical representations that depict the evolutionary relationships among diverse biological species or

entities based on their genetic, morphological, or behavioral characteristics. These trees are vital instruments in evolutionary biology, systematics, and comparative genomics, allowing researchers to infer the lineage and divergence of species over time. The construction of phylogenetic trees employs a variety of methods and algorithms, which can be broadly classified into Distance-Based Methods, Character-Based Methods, and Consensus Trees. Each branch of the tree represents a lineage, while the nodes signify divergence points where species have separated from a common ancestor. The length of the branches may indicate the extent of genetic change that has occurred over time.

Additionally, a clade is defined as a group of species that share a common ancestor. Phylogenetic trees are essential for visualizing and interpreting the evolutionary relationships among species (20, 27). By utilizing various construction methods and analytical techniques, researchers can derive significant insights into the history of life on Earth and the mechanisms of evolution. For instance, as illustrated in Figure 12, samples labeled PM 1071 and PM 1074 exhibit similarities to one another, sharing a common ancestor with PM 1073 and PM 1076.

To understand the value of the Hypericum plant and the need for a suitable method to identify the plant's authenticity, according to the statistics provided by the Financial Times website, the value of SJW is $2.68 of diluted earnings per share in 2023, surpassing $2.43 per diluted share in 2022, initial 2023 guidance of $2.40 to $2.50 and in line with the updated guidance of $2.65 to $2.70 (28). The market mentioned has shown a 36% growth in the first quarter of 2025 compared to the prior year.

Given the high economic value of Hypericum plants and the widespread buying and selling of supplements and raw materials derived from them, the deliberate or inadvertent identification of impurities is crucial. Additionally, various species and subspecies of Hypericum have distinct values based on the different amounts of active ingredients obtained from the extract of each species. Identifying these species is challenging because they are closely related to one another. Moreover, there is also the following admixture of dyes have been reported as co-occurring SJW adulterants: E123 Amaranth (FD&C Red #2), E133 Brilliant Blue (FD&C Blue #1), E110 Sunset Yellow (FD&C Yellow #6), and E102 Tartrazine (FD&C Yellow #5)with a spectrum close to Hypericum, which are sometimes used intentionally in adulterations. Therefore, identification techniques are of paramount importance. Furthermore, the reliability of detection, precision, and the economic feasibility of these methods should be thoroughly examined (29).

Traditional methods for identifying plant sample adulteration have several drawbacks. Among the drawbacks of traditional methods is their time-consuming nature. Many traditional methods require a considerable amount of time for identification, necessitating skills and advanced equipment. Some of these methods require skilled operators to handle sophisticated instruments, such as nuclear magnetic resonance (NMR) spectrometers and chromatography systems. Moreover, these older methods often only examine the plant itself, providing no information about its taxonomy and evolutionary history. Some methods are specific to a particular part of the sample, and the instrument used for detection may have limitations, for example, the ability to examine a sample in organic or aqueous solvents or under acidic conditions. Therefore, for specific samples or under certain conditions, the application of some devices may be restricted, and accurate identification may be challenging if the entire sample is not available, such as when only the root of a plant is accessible.

Nevertheless, the DNA-based methods address these shortcomings, making them

more suitable. There are newer methods that might also be beneficial, even possessing greater advantages, such as various RNA-based approaches and protein synthesis. In this context, the DNA barcoding method is deemed more suitable, given its capacity to operate on larger scales, transcending laboratory conditions and being applicable on a larger, industrial scale.

Since the genome is present in every cell that constitutes the plant, there is no limitation on the type of sample, allowing the use of any part of the plant. Additionally, by examining the genome of a plant, its family, subfamily, and phylogenetic relationships can be discerned and explored. While it is true that this method also requires complex instruments, it is relatively user-friendly, and its most crucial advantages lie in its exceptional accuracy and speed in advancing the work. Simplicity, cost-effectiveness, and accuracy make this method so useful (30-33). In Figure 1, the advantages and disadvantages of traditional and old methods, as well as the DNA barcode method, are presented in a classified manner.

This study represents a pioneering attempt to combine microscopic and DNA barcoding techniques for authenticating Hypericum species collected from various regions of Iran. The integration of molecular phylogenetics, using 18S rRNA, with bioinformatics analysis provides a robust framework for species identification. Unlike previous works focusing solely on morphology or chemical analysis, this work lays the foundation for a reproducible, scalable, and cost-effective authentication method suitable for both research and commercial applications. The insights gained from this analysis could be extended to identify adulteration in commercial SJW supplements, paving the way for stricter quality control standards in the herbal industry.

Despite the successful application of 18S rRNA gene sequencing in differentiating Hypericum species, the use of a single molecular marker may not capture the full spectrum of genetic diversity within this complex genus. Future studies could integrate additional barcoding loci such as matK, rbcL, or the internal transcribed spacer (ITS) regions to improve resolution and discriminatory power. Furthermore, expanding the geographical and taxonomic sampling to include more Hypericum species and subspecies from diverse climates would strengthen the phylogenetic analysis. Additionally, combining molecular data with metabolomic profiling (e.g., HPLC, LC-MS) may help correlate genetic differences with functional compound profiles, which is crucial for quality control in herbal medicine.

4. Conclusion

The use of the 18S rRNA gene as a barcode marker effectively distinguishes Hypericum species, enabling the construction of reliable phylogenetic trees. This method is a promising alternative to traditional morphological and chemical analyses, offering higher specificity and speed. Integrating this molecular approach with routine quality control practices can significantly enhance the authentication of Hypericum products, reduce the risk of adulteration, and ensure therapeutic efficacy.

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Authors' Contributions

MMZ: Resources, Conceptualization, Methodology, Project administration, Visualization, Funding acquisition, Supervision, Validation. EN: Investigation, Writing, Review, Data curation, Editing, Visualization, Formal analysis. NR: Investigation, Writing, Review, Data curation, Editing, Visualization, Formal analysis. SHM: Investigation, Writing, Review, Data curation, Editing, Visualization, Formal analysis. SKH: Investigation, Data curation, Formal analysis, Writing, Review, Editing. MHM: Resources, Conceptualization,

Methodology, Project administration, Visualization, Supervision, Validation.

Conflict of Interest

The authors declare that they have no conflict of interest.

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