DNA Barcoding of Two Thymelaeaceae Species: Daphne mucronata Royle and Thymelaea hirsuta (L.) Endl

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Abstract: Daphne mucronata Royle and Thymelaea hirsuta (L.) Endl both belong to the Thymelaeaceae family. Both species are used traditionally to treat several diseases along with various daily applications by Jordanian Bedouins. Traditionally, those species are identified through personal proficiency, which could be misleading due to human errors or lack of expertise. This study aims to investigate an effective DNA barcoding method to identify and characterize Daphne mucronata Royle and Thymelaea hirsuta plant species at the molecular level. Daphne mucronata Royle and Thymelaea hirsuta were collected from the ancient city of Petra in the Southern part of Jordan. Sequences of candidate DNA barcodes were amplified (rbcL, matK, and rpoC1), sequenced, and aligned to the blastn database. Moreover, the obtained sequences were compared with available sequences of related species at the GenBank database. Our results showed that DNA barcoding successfully identifies the two plant species using any of chloroplast genes (rbcL, matK, or rpoC1). The results emphasize the ability of DNA barcoding for identifying and characterizing different plant species through the recruitment of different barcode loci in molecular identification.

Keywords: DNA barcoding; Thymelaeaceae family; Daphne mucronata; Thymelaea hirsuta; matK; rbcL; rpoC1

1. Introduction

Thymelaeaceae family is a medium-sized family of Angiosperms that contains almost 898 species distributed in 50 different genera [1]. Daphne and Thymelaea genera comprise 95 and 30 species, respectively, representing around 23 percent of the family [2]. Thymelaeaceae family is widely used in folk medicine to treat several diseases as it has anti-leukemia, antitumor, anti-gout, anti-inflammatory, and antimicrobial pharmacological properties [3]. Among the Thymelaeaceae species are Daphne mucronata and Thymelaea hirsuta, with various medical and daily uses.

The Daphne mucronata Royle [4] is a wild evergreen shrub distributed in Southeast Asia, Afghanistan, Pakistan, Iran, North Africa, and South Europe [5]. Daphne mucronata is used in folk medicine to treat cancer, different skin disorders, ulcer, and purgative abortifacient [3,6–9]. Moreover, Daphne mucronata has analgesic, anti-inflammatory, and antimicrobial activities [10]. Recently, Daphne mucronata Royle showed a protective and anti-inflammatory effect on the stressed human adipose-derived mesenchymal stem cells protecting human adipose stem cells against monosodium iodoacetate and enhancing cell proliferation [11]. The phytochemical screening of Daphne mucronata Royle showed antimicrobial activity and antioxidant properties [12–15]. Moreover, ethyl acetate extract of Daphne mucronata aerial parts revealed the following chemical constituents: Coumarins, flavonoids, triterpenoids, diterpenes, lignin, and glucosides [10].

Thymelaea hirsuta (shaggy sparrow-wort or Mitnan in Arabic) is a xerophyte shrub that can grow up to two meters in height with a root system reaching up to 3.5 m depth,
and is known for its fleshy tiny size leaves and flowers [16]. *Thymelaea hirsuta* is a toxic plant with reported therapeutic properties [16]. Traditionally, the leaves of *Thymelaea hirsuta* were used to treat pinworms and skin conditions in the thirteenth century, while the bark was recruited to promote wound healing [16]. In addition, local Bedouins used the inner bark of *Thymelaea hirsuta* in manufacturing ropes and paper sheets [17,18]. Additionally, Bedouins have recruited powdered *Thymelaea hirsuta* in their traditional veterinary medicine to prevent miscarriages in she-camels [17]. Generally, steroidal compounds, flavonoids, coumarins, and lignans are the active chemical constituents that play a role in biological activity [19]. The *Thymelaea hirsuta* aqueous extracts are highly active sources of natural antioxidants, which play an essential role in controlling various pathological conditions, such as Parkinson’s disease and Alzheimer’s disease [20]. In addition, *Thymelaea hirsuta* plants’ aqueous extracts are rich in polyphenol contents that show antihypertensive and antidiabetic activities, thus the plant may be considered a food supplement for diabetic and hypertensive patients [21]. Furthermore, ethanolic extracts of *Thymelaea hirsuta* can significantly inhibit human adenocarcinoma cell growth [22]. Many *Thymelaea hirsuta* extract revealed antimicrobial and antifungal activities, and exhibited an excellent antioxidant activity [23]. Phytochemical screening of *Thymelaea hirsuta* aerial parts showed the presence of alkaloids tannins, saponins, steroids, coumarins, and anthraquinones [20]. Moreover, the aqueous extract of *Thymelaea hirsuta* revealed both hypoglycaemic and antidiabetic effects in normal glycaemic and induced diabetic rats, indicating the basis for *Thymelaea hirsuta* in diabetes treatment in Folk medicine [24]. In addition to the antidiabetic effect of *Thymelaea hirsuta* L. in a rat model, an antihypertensive effect was also reported [21]. In addition, *Thymelaea hirsuta* exhibited significant activity in acute inflammation compared to a standard anti-inflammatory drug (diclofenac) [25]. A recent study highlights the traditional usage of *Thymelaea hirsuta* extracts on cutaneous dermatophytosis and the new potential use of *Thymelaea hirsuta* as antiaging and better healing of the skin [26].

*Daphne mucronata* and *Thymelaea hirsuta* are essential as herbal medicine in folk remedies and traditional applications related to the daily life of Bedouins. The importance of both species inspires the research group to establish an effective DNA barcode to distinguish both species at the molecular level.

DNA barcoding is an identification tool of different samples based on the molecular marker of conserved regions [27,28]. DNA Barcoding is widely used to identify and classify animal and plant species; unknown samples even previously described [29,30]. Moreover, DNA barcoding is used for quality control and identification of food authentication, for example, seafood, herbal plants, and crops [31,32]. This study aims to use DNA barcoding to confirm the identity of the following two medicinal plant species: *Daphne mucronata* and *Thymelaea hirsuta* using *matK*, *rbcL*, and *rpoC1* genes as a barcode region.

2. Results

DNA was isolated, and targeted sequences were amplified using the selected PCR primers for the four barcode loci of *Daphne mucronata* and *Thymelaea hirsuta* (L.) Endl. DNA sequencing was successfully performed for 5 out of 6 loci in both selected plant species (Table 1). *Daphne mucronata* and *Thymelaea hirsuta* selected barcode regions were searched against the GenBank database [33]. Obtained sequences (Appendix A) were deposited at the GenBank database [33], and the deposited accession numbers are shown in Table 1. Barcode sequences were not retrieved for *Daphne mucronata* for the four selected barcode loci, while *Thymelaea hirsuta* retrieved sequences for only *matK* and *rbcL* (see retrieved accessions in Table 1). The obtained barcode sequences for *matK* and *rbcL* showed 97.96% identity for *matK* and 100% for *rbcL* of the retrieved two accessions of *Thymelaea hirsuta*. The obtained sequences of both species were aligned using a pairwise alignment search tool (Blastn). The two plant species showed 96% of identity for *matK*, and 99% for *rbcL*, as shown in Figure 1.
Table 1. The length of matK, rbcL, and rpoC1 barcode sequences in Daphne mucronata and Thymelaea hirsuta, along with the list of available sequences of Daphne mucronata and Thymelaea hirsuta that were retrieved from the GenBank database and our deposited sequences at GenBank [33].

| Plant Species         | Sequences Length (bp) | matK | rbcL | rpoC1 |
|-----------------------|-----------------------|------|------|-------|
|                       |                       |      |      |       |
| Daphne mucronata      |                       | 724  | 540  | -*    |
| Available GenBank accession number | N/A ** | N/A  | N/A  |
| Deposited accession number at GenBank | MZ851783 | OK188786 | N/A  |
| Thymelaea hirsuta     |                       | 685  | 682  | 479   |
| Available GenBank accession number | EU002191.1 | KY656740.1 | N/A  |
| Deposited accession number at GenBank | OK040774 | OK040775 | OK040776 |

* Unspecific amplification was obtained; ** N/A Unavailable at GenBank database.

The obtained sequences were run in blastn, and five high match scores were chosen to run phylogenetic analysis. The five related sequences were selected according to the highest BLAST hits. The retrieved genes of different species related to Daphne mucronata and Thymelaea hirsuta, along with E values, identity percentage, and the retrieved accessions, are shown in Table 2. Unavailable sequences (specific genes) for selected species was obtained by extracting the selected genes from the complete chloroplast genome via python code.
Table 2. The NCBI-BLAST results retrieved sequences of different species related to Daphne mucronata, sequence coverage (QC), E value, identity percentage, and retrieved accessions.

| Plant Species | Gene   | Related Species       | QC  | E-Value | Identity | Accession      |
|---------------|--------|-----------------------|-----|---------|-----------|----------------|
| Daphne mucronata | matK   | Daphne longilobata    | 98% | 0       | 99.16%   | MF786979.1     |
|               | matK   | Daphne tangutica      | 98% | 0       | 99.16%   | MH659257.1     |
|               | matK   | Daphne laureola       | 99% | 0       | 98.33%   | JN894978.1     |
|               | matK   | Daphne retusa         | 95% | 0       | 98.85%   | MH116619.1     |
|               | matK   | Daphne giraldii       | 98% | 0       | 98.04%   | MH659842.1     |
|               |        |                       |     |         |           |                |
|               | rbcL   | Daphne mezereum       | 100%| 0       | 99.44%   | KM360750.1     |
|               | rbcL   | Daphne longilobata    | 100%| 0       | 99.44%   | H849946.1      |
|               | rbcL   | Thymelaea hirsuta     | 100%| 0       | 99.07%   | Y15151.1       |
|               | rbcL   | Wikstroemia panpaninii| 100%| 0       | 99.07%   | MN722329.1     |
|               | rbcL   | Dirca occidentalis    | 100%| 0       | 98.52%   | MF963193.1     |
|               | rbcL   | Thymelaea hirsuta     | 100%| 0       | 97.96%   | EU002191.1     |
|               | matK   | Daphne laurolea       | 100%| 0       | 96.21%   | JN894952.1     |
|               | matK   | Daphne tangutica      | 100%| 0       | 96.36%   | MH659257.1     |
|               | matK   | Daphne mezereum       | 100%| 0       | 96.36%   | MF786979.1     |
|               | matK   | Daphne longilobata    | 100%| 0       | 95.77%   | JN894977.1     |
|               | rbcL   | Thymelaea hirsuta     | 99% | 0       | 100.00%  | KX527076.1     |
|               | rbcL   | Daphne mezeureum      | 99% | 0       | 99.62%   | AJ295262.1     |
|               | rbcL   | Stellera chamaejasme  | 99% | 0       | 99.62%   | AJ295262.1     |
|               | rbcL   | Wikstroemia monnula   | 99% | 0       | 99.62%   | AJ295262.1     |
|               | rpoC1  *| Daphne giraldii       | 97% | 0       | 99.15%   | NC_044085.1    |
|               | rpoC1  *| Daphne tangutica      | 97% | 0       | 99.15%   | NC_042950.1    |
|               | rpoC1  *| Stellera chamaejasme  | 97% | 0       | 99.15%   | NC_042714.1    |
|               | rpoC1  *| Daphne kiusiana       | 97% | 0       | 99.15%   | KY991380.1     |
|               | rpoC1  *| Daphne depauperate    | 97% | 0       | 99.15%   | MW245833.1     |

* Complete genome of chloroplast was found with an accession number then genes extracted by Python code.

The results show that the percentage identity range was the highest (99.16%) between Daphne mucronata matK, and both Daphne longilobata and Daphne tangutica. In comparison, the lowest percentage of identity was reported in Daphne mucronata matK barcode locus (98.04%) and Daphne giraldii species, belonging to the Thymelaeaceae family. The highest identity percentage was among Thymelaea hirsuta rbcL (100.00%) reported earlier in the database, followed by 99.26% found in Daphne mezereum rbcL, Stellera chamaejasme rbcL, and Wikstroemia monnula rbcL (Table 2).

The top five related sequences that appeared in Table 2 were recruited in phylogenetic trees construction using Mega X software shown in (Figure 2). Figure 2 shows phylogenetic trees of Daphne mucronata related species using matK, and rbcL barcode loci. The matK barcode could discriminate Daphne mucronata from other related species (Figure 2A), while rbcL can discriminate between Daphne mucronata and Daphne mezeureum, Daphne laurolea, Dirca occidentalis, and Thymelaea hirsuta (Figure 2B). In Figure 2, phylogenetic trees of Thymelaea hirsuta and other related species show that matK can discriminate between Thymelaea hirsuta, Daphne laurolea, and Daphne mezeureum (matK, rbcL, and rpoC1) barcode loci (Figure 2C). While Figure 2D shows that rbcL can discriminate between Thymelaea hirsuta and the five related species. The rpoC1 can discriminate between Thymelaea hirsuta and Stellera chamaejasme (Figure 2E). Further analysis was performed through the NCBI-Taxonomy browser to check the ability of the obtained sequences to fit within the proper plant family (Thymelaeaceae). Table 3 shows the number of obtained hits (organisms) according to the taxonomy browser (NCBI), once running sequences through blastn (NCBI).
database. In Table 3 the NCBI taxonomy Entrez results of the retrieved lineage hits support that all sequences are able to be discriminated and retained to Thymelaeaceae family.

Figure 2. The phylogenetic trees (Neighbor-Joining method) of the top five related species and obtained barcode sequences of Thymelaea hirsuta and Daphne mucronata. (A) Daphne mucronata matK, the sum of branch length is 0.02958153; (B) Daphne mucronata rbcL, the sum of branch length is 0.02199074; (C) Thymelaea hirsuta matK, the sum of branch length is 0.05720029; (D) Thymelaea hirsuta rbcL, the sum of branch length is 0.01331361; (E) Thymelaea hirsuta rpoC1, the sum of branch length is 0.50635593.
Table 3. NCBI taxonomy Entrez results; running obtained sequences via blastn and retrieving the lineage hits and number of aligned sequences related to Thymelaeaceae family.

| Sequence (Organism) | Taxonomy               | Number of Hits | Number of Organisms |
|---------------------|------------------------|----------------|---------------------|
| matK (Daphne Mucronata) | Thymelaeaceae          | 104            | 32                  |
| rbcL (Daphne Mucronata) | Thymelaeaceae          | 119            | 66                  |
| matK (Thymelaea hirsuta) | Thymelaeaceae          | 105            | 32                  |
| rbcL (Thymelaea hirsuta) | Thymelaeaceae          | 118            | 66                  |
| rpoC1 (Thymelaea hirsuta) | Thymelaeaceae          | 101            | 43                  |

3. Discussion

Jordanian Flora is rich with an enormous variety of plant species belonging to 112 plant families, where more than 363 species are considered medicinal due to their therapeutic activity [34–36]. In Jordan, the Thymelaeaceae family is represented by two genera Daphne (Daphne mucronata Royle) and Thymelaea (three species; Thymelaea hirsuta, Thymelaea passerine, and Thymelaea pubescens) [37]. Daphne mucronata is distributed in Petra, Karak, Ma’an, and Tafila [38]. At the same time, Thymelaea hirsuta is distributed in the southern part of Jordan (Petra, Tafila, Shobak, and Ma’an) [37,38]. The usage of both selected species in folk medicine and the recruitment of Thymelaea hirsuta in Bedouins’ daily life makes both species excellent candidates for molecular identification (barcoding).

Much research was conducted to investigate the therapeutic and antioxidant activities of both Daphne mucronata and Thymelaea hirsuta. However, molecular identification and phylogenetic characterization were very limited. Exploring the GenBank database for Daphne mucronata retrieved no results [33], indicating that our obtained sequences are new and firsthand. At the same time, Thymelaea hirsuta search retrieved deposited sequences for both rbcL and matK sequences but nothing for both rpoC1 [39]. The length of gene sequences is within the average length, satisfying the previously reported criteria [40]. In addition, DNA barcoding was successfully identified Thymelaea hirsuta and Daphne mucronata species. A total of 5 sequences were successfully obtained for the two plant species using different chloroplast barcode loci (rbcL, matK, and rpoC1). Among those sequences, about 3 novel sequences were not included earlier within the GenBank database (OK188786, OK040775, OK040776). Moreover, the identity percent between our Thymelaea hirsuta sequence and previously deposited sequence in GenBank database is 97.96% for matK and 100.00% for rbcL.

The Molecular phylogenetic relationships of different species from Thymelaeaceae family sequences from Africa and Australia were investigated earlier by parsimony analysis [41], including Thymelaea hirsuta Endl (the original sequence was obtained from [42]). The van der Bank study was limited to rbcL, trnL intron, and trnL-F intergenic spacer sequences, and separate sequence analysis of the selected sequences produced nonidentical phylogenetic outcomes. Meanwhile, combined sequences analysis did improve the resolution of phylogenetic discrimination among different clades [41]. Furthermore, Daphne mucronata sequences were not included in the study mentioned above [41]. In another recent study, phylogenetic analysis using maximum parsimony and Bayesian inference of the internal transcribed spacer (ITS) and rbcL, trnL intron, and trnL-F intergenic spacer revealed that the Thymelaeaceae is not a monophyletic family [43]. The discrimination capacity of matK, rbcL, and rpoC1 barcode regions were divergent among studied species, indicating that each species could recruit different locus (loci), in terms of identification and molecular characterization. However, the discrimination capacity of rpoC1 as a candidate barcode region is limited and needs future study. Lower discrimination capacity of rpoC1 compared with matK and rbcL is probably due to limited sequences availability in reference databases for rpoC1, which lead to low identification capacity [44]. Many studies in plant DNA barcoding used matK and rbcL genes as barcode regions. Further studies should be done using other barcode genes, as there is no universal primer found effective in
plants. DNA barcoding can be used to identify plant species, specifically medicinal plants. Further research should be carried out to establish a complete DNA barcodes database of all medicinal plants.

4. Materials and Methods

Fresh leaves of the two selected species from the Thymelaeaceae family (Daphne mucronata and Thymelaea hirsuta (L.) Endl) were collected from the ancient city of Petra (Jordan) (Locality: 30.324181945297152, 35.47997922146477). Samples collection was conducted via a specialized plant taxonomist [37]. Stored leaves were ground using liquid nitrogen, and DNA was extracted using commercial kits (Qiagen). DNA quality and quantity were checked spectrophotometrically and via 1% gel electrophoresis before the PCR amplification. Different Chloroplast loci (matK, rbcL, and rpoC1) were amplified using the following primers: matK (Forward—CCCRTYCATCTGGAAATCTTGGTTC and reverse—GCTRTRATAATGAGAAAGATTTCTGC) [45], rbcL (Forward—TGTCACCACAAACAGAAAC and reverse—TCCGATGACCTGCAGTACG) [46], and rpoC1 (—GGCAAAGAGGGAAGTTTCG and reverse—CCATAAGCATATCTTGAGTITG) [47]. PCR amplifications were conducted using 5× HOT FIREPol® Blend master mix; Initial denaturation (5 min, 95 °C), followed by 40 cycles of denaturation (30 s, 95 °C), annealing (30 s at 54 °C). The final extension cycle (30 s at 72 °C) was applied for all PCR reactions, and amplified DNA fragments were qualitatively checked via Agarose gel electrophoresis before sequencing. The Amplified fragments were purified and sequenced using Sanger sequencing method (ABI PRISM® kit, Macrogen company, Korea). Chromatograms were analyzed using FinchTV software [48], and obtained sequences were further analyzed using the NCBI-BLAST online tool [49] to check related sequences in the nucleotide database. Furthermore, five related sequences with a high matching score were obtained from NCBI-GenBank Entrez for further phylogenetic analysis for each plant sample. Corresponding genes were extracted using python code for species with complete chloroplast genomes [50]. Neighbor-joining phylogenetic trees were constructed using MEGA X software [51] to evaluate the phylogenetic relationships and the effectiveness of barcode discrimination at the species level. Obtained sequences were further analyzed using the NCBI taxonomy database (Lineage), via counting the number of (hits) organisms along appeared in taxonomy browser, once running the obtained sequences through NCBI blastn.

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Conflicts of Interest: The authors declare no conflict of interest.
Appendix A

Table A1. List of obtained plant samples sequences in FASTA format.

| Seq | Organism          | Accession Number | Gene         | Partial CDS | FASTA Sequence |
|-----|-------------------|------------------|--------------|-------------|----------------|
| Seq1 | Daphne mucronata  | MZ851783         | matK         | partial cds | >seq1 | AAAGTGATTT TTTTGCATT TTATAGGCGTT TTTTTTTTCT ACGAGTATTT AAATTTGAAG 60 |
|     |                   |                  | rbcL         | partial cds | >seq2 | AATTGACTTA TTATACTCCT GAATATGAAA CCAAAGATAC TGATATCTTG GCAGCGTTCC 60 |
| Seq2 | Thymelaea hirsuta | OK040774         | matK         | partial cds | >seq3 | CTTACGAGTTAT TTATAATTTGA AGAGTCTTAG TACTTCACAA AAATGCATTT CGATTTTGAA 60 |
|     |                   |                  | rbcL         | partial cds | >seq4 | AGAGTATAAA TTGACTTATT ATACTCCTGA ATATGAAACC AAAGATACTG ATATCTTGGC 60 |
| Seq3 | Thymelaea hirsuta | OK040775         | rpoC1        | partial cds | >seq5 | GATCATACGG CGCGTTCTGTC ATTGTTGTTG GCCCCTCACT TTCATTACAT CGCTGTGGGT 60 |

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30. Raupach, M.J.; Barco, A.; Steinke, D.; Beermann, J.; Laakmann, S.; Neumann, H.; Kihara, T.C.; Pointner, K.; Radulovic, I. The application of DNA barcodes for the identification of marine crustaceans from the North Sea and adjacent regions. *PLoS ONE* **2015**, *10*, e0139421. [CrossRef] [PubMed]

31. Galimberti, A.; Labra, M.; Sandionigi, A.; Bruno, A.; Mezzasalma, V.; De Mattia, F. DNA barcoding for minor crops and food traceability. *Adv. Agric.* **2014**, *2014*, 831875. [CrossRef]

32. Khaksar, R.; Carlson, T.; Schaffner, D.W.; Ghorashi, M.; Best, D.; Jandhyala, S.; Traverso, J.; Amini, S. Unmasking seafood mislabeling in US markets: DNA barcoding as a unique technology for food authentication and quality control. *Food Control* **2015**, *56*, 71–76. [CrossRef]

33. GenBank [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information. 2013. Available online: https://www.ncbi.nlm.nih.gov/genbank/ (accessed on 7 July 2021).

34. Taifour, H.; El-Oqlah, A.; Ghazanfar, S. *The Plants of Jordan: An Annotated Checklist*; Kew Publishing: London, UK, 2017.

35. Oran, S.; Al-Eisawi, D. Check-list of medicinal plants in Jordan. *Dirasat* **1998**, *25*, 84–112.

36. Oran, S.A. The status of medicinal plants in Jordan. *J. Agric. Sci. Technol. A* **2014**, *4*, 461–467.

37. Zohary, M. *Flora Palaestina*; Israel Academy of Sciences and Humanities: Jerusalem, Israel, 1966.

38. Taifour, H.; El-Oqlah, A. *Jordan Plant Red List*; Royal Botanic Garden: Amman, Jordan, 2015; Volume 1.

39. NCBI. 2021. Available online: https://www.ncbi.nlm.nih.gov/nuccore/?term=Thymelaea+hirsuta (accessed on 7 July 2021).

40. Kress, W.J.; Erickson, D.L. DNA barcodes: Genes, genomics, and bioinformatics. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 2761–2762. [CrossRef]

41. Van der Bank, M.; Fay, M.F.; Chase, M.W. Molecular phylogenetics of Thymelaeaceae with particular reference to African and Australian genera. *Taxon* **2002**, *51*, 329–339. [CrossRef]

42. Fay, M.F.; Bayer, C.; Alverson, W.S.; de Bruijn, A.Y.; Chase, M.W. Plastid rbcL sequence data indicate a close affinity between Diegodendron and Bixa. *Taxon* **1998**, *47*, 43–50. [CrossRef]

43. Beaumont, A.J.; Edwards, T.J.; Manning, J.; Maurin, O.; Rautenbach, M.; Motsi, M.C.; Fay, M.F.; Chase, M.W.; Van Der Bank, M. *Gnidia* (Thymelaeaceae) is not monophyletic: Taxonomic implications for Thymelaeoideae and a partial new generic taxonomy for Gnidia. *Bot. J. Linn. Soc.* **2009**, *160*, 402–417. [CrossRef]

44. Kolter, A.; Gemeinholzer, B. Plant DNA barcoding necessitates marker-specific efforts to establish more comprehensive reference databases. *Genome* **2021**, *64*, 265–298. [CrossRef]

45. Yu, J.; Xue, J.H.; Zhou, S.L. New universal matK primers for DNA barcoding angiosperms. *J. Syst. Evol.* **2011**, *49*, 176–181. [CrossRef]

46. Fay, M.F.; Swensen, S.M.; Chase, M.W. Taxonomic affinities of *Medusagyne oppositifolia* (Medusagynaceae). *Kew Bull.* **1997**, *52*, 111–120. [CrossRef]

47. Sass, C.; Little, D.P.; Stevenson, D.W.; Specht, C.D. DNA barcoding in the cycadales: Testing the potential of proposed barcoding markers for species identification of cycads. *PLoS ONE* **2007**, *2*, e1154. [CrossRef]

48. FinchTV 1.4.0; Geospiza, Inc.: Seattle, WA, USA, 2006; Available online: http://www.geospiza.com (accessed on 7 July 2021).

49. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]

50. Awad, M.; Fahmy, R.M.; Mosa, K.A.; Helmy, M.; El-Feky, F.A. Identification of effective DNA barcodes for Triticum plants through chloroplast genome-wide analysis. *Comput. Biol. Chem.* **2017**, *71*, 20–31. [CrossRef] [PubMed]

51. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef]