DNA Barcoding of *Masdevallia* Orchids Using the *matK* Gene

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Abstract:

Maintaining global biodiversity is becoming more of a focus as the quality of this biodiversity declines. Conservation efforts need to be targeted at areas where this loss of biodiversity is most critical. Orchids are a family of plant facing significant survival pressures. *Masdevallia* is a genus of neotropical orchids which is poorly represented in orchid studies. When not flowing, individuals of this genus are morphologically indistinguishable from each other. DNA barcoding will assist in targeting these efforts by genetically identifying unknown species in threatened ecosystems. Many different loci in the orchid genome have been examined for use as a barcode, and the matK locus has had the best results. The objective of this study is to use the matK locus to create a DNA barcoding system which distinguishes between individuals of the *Masdevallia* genus. DNA has been isolated from samples and PCR has been done to amplify the *matK* locus. PCR products were sequenced using ABI sequencing, and the resulting sequences were aligned to create a phylogenetic tree. This tree contains unedited sequencing data, so while not conclusive, it indicates that this DNA barcoding system is sufficient to distinguish between samples at the species level. This will contribute to a DNA library, so unknown orchid individuals may be better identified in threatened ecosystems.

Keywords

DNA Barcode : *Masdevallia* : Conservation : matK : Orchid

Introduction

The state of the worlds biodiversity is being challenged. There is currently a substantial and rapid decline in global biodiversity; this process is being referred to as the sixth mass extinction (Bellard et al., 2012). To prevent further declines and preserve what is left, significant conservation efforts need to be conducted. To ensure continued survival of a species, it is imperative that the genetic diversity of said species remain intact, as genetic diversity directly relates to the ability of a species to survive environmental pressures. This will require both ecological and genetic studies, as well as *ex situ* or *in vitro* propagation of a species, and subsequent reintroduction of that species into an ecosystem (Seaton et al., 2010). Conservation efforts should therefore be targeted to areas of failing biodiversity. To establish the biodiversity of an ecosystem, species need to be quantified. To quantify species, distinctions between species need to be determined. It is common practice to use morphological features when grouping individuals found in an ecosystem into species (**Balram et al. 2004)**. However, in the case of some plants, this is inadequate as they are morphologically indistinguishable. Alternative taxonomical methods must then be established.

Orchidaceae as a family have suffered a global decline in biodiversity due to environmental pressures such as habitat loss and climate change. Orchids are a prime candidate for conservation efforts (Koopowitz, 1993). When some species of orchids are not flowering, they are morphologically indistinguishable from each other. Using morphology to evaluate orchid biodiversity is inadequate. Instead, the use of DNA barcoding can be implemented.

DNA barcoding is a taxonomical method which assists with the identification of species. It makes use of a locus common to all species that has high levels of variability outside of a species group, and low levels of variability within a species. This effectively groups individuals into species based on the level of variability at the selected locus. For a locus to be effective as a barcode, it must be common for

all species, small enough to allow for amplification and sequencing, and have interspecific variation that is greater than its intraspecific variation. Additionally, conserved flanking regions on either side of the locus which allow for universal amplification of all biological samples is necessary, especially when dealing with an unknown sample (Cowan and Fay, 2012**, Parveen et al. 2012**). Animals have a singular locus which allows for universal barcoding. The cytochrome oxidase1 ge ne of animal mitochondrial DNA is effective for differentiating all animal species (Erickson et al., 2008). Plants, orchids included, do not have a similar locus. Instead, there are a handful of loci that have been studied as DNA barcodes, and they can be used in combination to differentiate species. Within orchid nuclear DNA, Internal Transcribed Spacer (ITS) loci have been evaluated as potential barcodes. Within orchid chloroplast DNA, rpoB and rpoC1 (RNA polymerase Subunits), rbcL (Rubisco Large Subunits), and matK (MaturaseK) genes have held potential as barcoding loci. These have been evaluated in terms of amplification and sequencing success, as well as the ability of the locus to differentiate between species. From these potential barcodes, matK shows the most promise as an effective barcode. It is easily amplified and sequenced, and most effectively differentiates between species (Ali et al., 2014, Parveen et al. 2012).

Masdevallia is a neotropical genus of the family *Orchidaceae*. This genus lacks DNA barcoding information. There are over 500 species of *Masdevallia*, however there are only 16 species with barcodes represented in the "Barcode of Life Database" **(BOLD Systems**). While this database does not make up all the barcoding information of *Masdevallia*, it is a good representation of how much is known about *Masdevllia* barcodes in comparison to how much is not known. This study aims to contribute towards barcoding the *Masdevallia* genus, as it significantly lacks barcoding information.

Here we have created a DNA barcoding system that differentiates between species of the genus *Masdevallia***.** This barcoding system makes use of the *matK* locus of the orchid genome. Samples from different individuals of the genus *Masdevallia* were taken and DNA from these samples was extracted. PCR reactions amplifying the matK locus will be conducted and resulting PCR products were evaluated

for successful smplification via gel electrophoresis. Successful PCR products were sent for sanger sequencing, and sequencing data was used to construct a phylogenetic tree. This allows for more concrete understanding of evolutionary relationships between species of the genus *Masdevallia*. A DNA barcoding library for the genus *Masdevallia* can be created from this information, which will allow for the evaluation of biodiversity within ecosystems so that conservation efforts may be applied.

Materials and Methods:

Masdevallia samples were taken from the collection owned and maintained by the Orchid Species Preservation Foundation (OSPF). This collection is housed at the Muttart Conservatory in Edmonton, Alberta. Leaf samples were clipped from individual plants, incorporating ethanol to maintain sterol conditions. These were then placed in bags and cataloged, and this record was maintai ned throughout the study.

Processing of the samples began in the lab. Each leaf was hole punched using a standard classroom hole puncher as many times as the leaf size will allow. By cutting samples into uniform discs, this allowed for a consistent quantity of genetic material to be maintained during extraction and subsequent PCR (4 discs are roughly 150mg of tissue). Discs were then stored in 15 mL plastic conical tubes at -80 degrees Celsius until DNA extraction was performed.

DNA extraction began with the creation of a tissue homogenate. Four discs from each sample was taken out of storage, and cut in half using a scalpel. Samples were then exhibited to mechanical forces via Zymo BeadBashing Lysis system. Samples 1-14 underwent bashing in the presence of lysis buffer, at the hand of a Terralyzer, for 2 minutes. Samples 15-23 were first cut into many small pieces using a scalpel, before being bashed with a genie vortexer for 12 minutes in the presence of beads and lysis buffer. Tissue homogenates then underwent miniprep via the Zymo Research Plant and Seed

MiniPrep kit. Extracted DNA was quantified and tested for purity using the NanoVue Plus UV spectrophotometer. Any remaining tissue samples are kept in storage, frozen at -80 °C.

Polymerase Chain Reactions (PCR) was setup to amplify the matK locus. The forward primer for matK, coding 5'-CGTACAGTACTTTTGTGTTTACGAG-3' was used in combination with the reverse primer 5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3' (Cuénoud, 2002). These primers can be seen outlined in table 1. PCR reactions were conducted with a total volume of 50ul. The total reaction volume is broken down into 1.25 units PrimeSTAR GXL DNA Polymerase (Takara), 1X PrimeSTAR GXL buffer (Takara), 200 uM dNTP mixture, 1 uM of both forward and reverse primer, and 100 ng of template DNA. Reactions were carried out using the BioRad C1000 ThermoCycler. This cycles reaction temperatures 30 times after an initial denaturation step for 10 seconds at 98˚C. Each cycle included a denaturation at 98 ˚C for 10 seconds, followed by an annealing step at 55˚C for 15 seconds and a subsequent elongation step at 68˚C for 60 seconds. The final cycle ended with a 2-minute elongation step at 68˚C.

The resulting PCR product was then run on gel electrophoresis to determine success. Banding was expected to be singular and at 900bp on the gel. A 20-lane gel was used with a 1.2% agarose gel composition in a 0.5X TBE running buffer. Gels were run for 1.5 hours at 90V, or until the DNA was roughly half way down the gel. **S**amples that demonstrated singular banding at 900bp were determined to have amplified *matK* sequence.

The PCR products containing *matK* sequence were then put through a clean-up procedure to remove excess dNTPs, DNA pol, or primers that may interfere with sequencing. This was done using a Nucleospin Gel and PCR Clean-up Kit by Macherey-Nagel. Purified samples were measured for concentration and purity using the NanoCell spectrophotometer before being set up for sequencing. Sequencing reactions were arranged at a total volume of 10uL: 9uL of part DNA template and milliQH_2O, and 1uL of either forward or reverse *matK* primer. Stock primer solutions were diluted by a factor of

0.125 before use in sequencing reactions, and both forward and reverse primers were used for each sample, providing 2X coverage of each sequence. Reactions were then sent to the University of Alberta for ABI sequencing. The resulting sequencing data was then compared and manipulated using CLC Viewer. This allowed for tree building, which can be seen in Figure 2**.**

Results:

A total of 23 samples were collected from the OSPF at the Muttart Conservatory, representing a total of 15 different species. DNA was then extracted from these samples and tested for concentration and purity via spectrophotometry. All 23 samples with their DNA extraction concentrations and purities can be seen in Table 2. Extraction concentrations range from 1.6ng/uL to 20.0ng/uL and A260/A280 rations range from 0.449 – 1.395. Additional samples that had DNA extraction procedures already conducted on them were then incorporated into procedures. These samples were taken, and DNA was isolated by Alex Worthy in 2016. The samples and their DNA concentrations and purities can be seen in Table 3. There is a total of 32 samples representing 24 different species. DNA concentrations range from 11.2ng/uL to 35.5ng/uL and A260/A280 ratios range from 1.053 – 1.542. Samples were then amplified by polymerase chain reaction (PCR), and the resulting PCR products were analyzed via gel electrophoresis. Primers used can be seen in Table 1, and banding was expected from these primers around 900bp for the *matK* locus. Gel images can be seen in Figure 1. From panels (A), (B), (C), and (D), samples 2, 24, and 39 have weak banding. All other reactions have singular banding of an adequate single strength. Controls from these lanes are free of contamination. From panel (D), samples 24, 48, 49, 50, 51, 52, and 55 have weak banding singles, however signals are present.

PCR reactions were then cleaned to purify DNA away from contaminants such as DNA pol, dNTPs, or excess primer. All DNA concentrations and purities can be seen in Tables 4 and 5. DNA

concentrations range from 4.2ng/uL to 51.5ng/uL and A260/A280 ratios range from 1.646 – 2.070. Cleaned PCR reactions were then sent for sequencing. Unedited sequences were aligned, and those alignments were built into phylogenetic trees using the program CLC Viewer. Trees from samples 1-23 can be seen in Figure 1.

Discussion:

Of the total 55 samples, 23 had DNA isolated as a part of this project. DNA concentrations of these isolations had a large range from 1.6 – 20.0ng/uL. This is due primarily to differences in leaf structure. While the total amount of leaf tissue used in each extraction was controlled for by using 4 discs of a uniform size, both the thickness and cuticle type of the leaf, which the discs were cut from, differed. Some leaves were thicker than others, and some leaves had a waxier cuticle than others. Both of these factors influence how much or how easily DNA could be extracted, and the range of DNA concentrations reflect that. This range is comparable to other work with *Masdevallia* done by Alex Worthy in 2016. Those previous findings can be seen in Table 3. The A260/A280 absorbance ratios also have a large range from 0.449 – 1.395. Pure DNA has a ratio within 1.8 – 2.0. While the DNA isolated here is impure, it is comparable to past findings in *Masdevallia* research which can be seen in Table 3. Impurities are likely plant based contaminants and did not interfere with subsequent PCR reactions.

PCR reactions done to amplify the *matK* locus were conducted using template DNA from these isolates. It is here that isolations from previous work done by Alex was included into this procedure. All bands were singular in nature and banding occurred at around 900bp, which indicates successful *matK* amplification. This can be seen in Figure 1. Sample 2, *M. picca* 11106A, had weak banding on the gel, indicating a failed PCR amplification. This could be because the *matK* primers are inadequate for amplification within this species. This can be seen in Figure 1(A). Sample 15, *M. revoluta* 11628C, was

not included in PCR reactions because the concentration of DNA isolated from tissue samples was too low for amplification. Sample 15 was the first sample to have tissues homogenized with the genie vortexer, and this may explain the low DNA yield. Sample 24, *M. aenigma* 11111A, was amplified twice. The first amplification can be seen in Figure 1 (C), and the second in Figure 1 (E). This species failed to amplify on both occasions, and it is possible that the recorded concentration of this sample is not reflective of its actual concentration. The concentration of sample 24 should be re-established. Figure 1 (E) has many low signal bands, and a slight curve in the banding pattern. To make the 1.2% agarose gel used in this image, 0.5X TBE running buffer which had been used previously was incorporated into the gel matrix. This could explain the curve to the gel. The low signal bands are likely a product of frozen samples not being thawed correctly. Extractions are frozen before they are ready for PCR, and if they are not mixed well there could be a lower than anticipated template amount incorporated into PCR reactions, which would result in low amplification levels. This would explain weak signal strength of bands in Figure 1 (E). PCR clean-up results support this speculation. Samples depicted in Figure 1 (E) have lower concentration post PCR clean-up. This can be seen in Table 5. Sequencing is partially dependent on template DNA volume, and sequence results will determine if volumes were adequate. Samples 24-55 have not been sequenced.

Sequencing results from samples 1-23 can be seen in a phylogenetic tree in Figure 2. It is important to make note that the sequences used to create these phylogenetic trees are entirely unedited. Because of this, the accuracy with which each species is placed in relation to each other will be adversely affected. However, there is some degree of barcoding success, as species such as *M. porphyrea, M. polysticta var alba,* and *M. pinocchio* can be seen grouped together with other like species and these placements have high bootstrap values. Additionally, both the forward and reverse primers have created trees that are comparable, indicating that sequences have been done so correctly. More work is required to edit sequences and resolve sequence ambiguities. Creating a phylogenetic tree

from edited sequences would more accurately represent sequence data and could resolve ambiguities between forward and reverse primer trees. Additionally, sequencing could be done for samples 24-55, the resulting data edited, and then included into a phylogenetic tree with samples 1-23. This would conclusively demonstrate whether the DNA barcoding system built around the *matK* locus was sufficient in determining sample resolution at the species level. Early data which makes use of unedited sequences from samples 1-23 indicates this system to be successful, however more sequencing and editing is necessary to make these findings conclusive.

This research will contribute DNA barcoding information to a large DNA barcoding library, such as BOLD Systems, and is critical as *Masdevallia* are underrepresented in this database. Additions to this database will provide a better understanding of evolutionary relationships of the species in this genus. Conservation efforts directed at preserving *Masdevallia* orchids could make use of this DNA barcoding library to evaluate the biodiversity of orchids in an ecosystem, and then target such efforts to areas of declining biodiversity. Further sample additions will enrich this database, as this project is ongoing. The addition of more sequence information will improve the quality of the database and its ability to distinguish between species.

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Tables and Figures:

Table 1. Primer type and sequence.

***matK* Primer Direction**	**Primer Name**	**Sequence (5'-3')**
Forward Primer	*matK* 3F_KIMf	CGTACAGTACTTTTGTGTTTACGAG
Reverse Primer	*matK* 1R_KIMr	ACCCAGTCCATCTGGAAATCTTGGTTC

Table 2. **Samples taken from OSPF collection at the Muttart Conservatory and DNA concentration/purity after extractions.**

Sample #	Species	OSPF #	Concentration (ng/uL)	Absorbance A260/A280
1	*M. patula*	11018B	7.4	0.937
2	*M. picca*	11106A	14.6	1.187
3	*M. patula*	10988A	9.2	1.039
4	*M. polysticta var alba*	15719A	18.0	1.395
5	*M. polysticta*	17102K	15.0	1.211
6	*M. pinocchio*	14982C	12.9	1.127
7	*M. posadae*	16389D	10.3	1.079
8	*M. x mystica*	17103F	19.0	1.301
9	*M. peristeria*	10819B	7.6	1.041
10	*M. rigens*	17072M	9.4	1.027
11	*M. polysticta f hoja fina*	11605A	7.1	0.922
12	*M. polysticta f hoja fina*	15113B	20.0	1.223
13	*M. revoluta*	11623B	4.2	0.724
14	*M. persicina*	15724C	17.0	1.349
15*	*M. revoluta*	11628C	1.6	0.449
16*	*M. porphyrea*	16802H	4.6	1.011
17*	*M. porphyrea*	15721F	7.2	1.059
18*	*M. pinocchio*	16338D	7.3	1.394
19*	*M. caesia*	16788D	7.2	1.117
20*	*M. porphyrea*	11045D	15.6	1.279
21*	*M. princeps*	11154B	5.1	0.944
22*	*M. peristera*	16994F	10.2	1.115
23*	*M. porphyrea*	10839A	17.0	1.185

* indicates samples for which the genie vortexer was used

** pure DNA has an absorbance ratio between 1.8 – 2.0

Table 3. **DNA concentrations/purity from samples incorporated from past research.**

Sample #	Species	OSPF ID	[DNA] (ng/µL)	A260/A280
24	*M. aenigma*	11111A	17.0	1.255
25	*M. agaster*	11030A	24.0	1.333
26	*M. albella*	12429A	35.5	1.365
27	*M. andreettaeana*	11128A	12.6	1.082
28	*M. andreettaeana*	11066B	17.0	1.283
29	*M. andreetaeana*	11579C	21.5	1.319
30	*M. angulata*	11582H	27.0	1.500
31	*M. angulata*	11093B	22.5	1.490
32	*M. angulata*	10887B	14.5	1.189
33	*M. antonii*	12430A	25.5	1.378
34	*M. asterotricha*	10816A	26.0	1.300
35	*M. ayabacana*	10990D	15.2	1.196
36	*M. ayabacana*	10868C	19.0	1.242
37	*M. ayabacana*	11078G	15.6	1.376
38	*M. ayabacana*	11152B	17.5	1.167
39	*M. bennettii*	11581D	20.5	1.429
40	*M. bennettii*	10745A	18.5	1.345
41	*M. bennettii*	10746B	19.5	1.383
42	*M. cuprea*	10744A	20.0	1.053
43	*M. ignea*	11327A	15.8	1.255
44	*M. ignea*	11190B	24.5	1.361
45	*M. klabochorum*	10890A	18.5	1.542
46	*M. leptoura*	11008B	24.0	1.412
47	*M. norae*	11632A	26.0	1.333
48	*M. bicolour*	11598E	17.0	1.197
49	*M. bicolour*	11163B	19.5	1.300
50	*M. bidenta*	11586A	21.5	1.352
51	*M. bonplondi*	10780A	21.5	1.265
52	*M. buccinator*	11600C	26.0	1.238
53	*M. bulbophyllopsis*	10775A	11.2	1.295
54	*M. bulbophyllopsis*	11591C	22.5	1.250
55	*M. cacodes*	11592C	19.5	1.287

* Samples collected, and DNA extracted by Alex Worthy, 2016.

Table 4. **DNA concentration and purity of cleaned up PCR reactions for samples 1-23.**

Sample #	Species	OSPF #	Concentration (ng/uL)	Absorbance A260/A280
1	*M. patula*	11018B	30.0	1.714
2	*M. picca*	11106A	N/A	N/A
3	*M. patula*	10988A	24.0	1.745
4	*M. polysticta var alba*	15719A	39.5	1.756
5	*M. polysticta*	17102K	44.0	1.796
6	*M. pinocchio*	14982C	23.5	1.728
7	*M. posadae*	16389D	28.5	1.717
8	*M. x mystica*	17103F	30.0	1.765
9	*M. peristeria*	10819B	28.0	1.879
10	*M. rigens*	17072M	24.5	1.899
11	*M. polysticta f hoja fina*	11605A	33.0	1.833
12	*M. polysticta f hoja fina*	15113B	35.5	1.919
13	*M. revoluta*	11623B	37.5	1.875
14	*M. persicina*	15724C	28.5	1.827
15	*M. revoluta*	11628C	N/A	N/A
16	*M. porphyrea*	16802H	35.5	1.868
17	*M. porphyrea*	15721F	38.5	1.878
18	*M. pinocchio*	16338D	33.5	1.811
19	*M. caesia*	16788D	29.0	1.801
20	*M. porphyrea*	11045D	30.0	1.765
21	*M. princeps*	11154B	38.0	1.810
22	*M. peristera*	16994F	23.5	1.843
23	*M. porphyrea*	10839A	24.5	1.782

* pure DNA has an A260/A280 ratio of 1.8 – 2.0

** N/A indicate samples that were not suitable for PCR clean-up due to failed extraction (15) or failed PCR (2)

Table 5. **DNA concentration and purity of cleaned up PCR reactions for samples 24-55.**

Sample #	Species	OSPF ID	Concentration (ng/uL)	Absorbance A260/A280
24	*M. aenigma*	11111A	19.5	1.646
25	*M. agaster*	11030A	31.0	1.824
26	*M. albella*	12429A	40.0	1.818
27	*M. andreettaeana*	11128A	29.5	1.815
28	*M. andreettaeana*	11066B	42.0	1.826
29	*M. andreetaeana*	11579C	37.0	1.850
30	*M. angulata*	11582H	32.0	1.829
31	*M. angulata*	11093B	45.5	1.820
32	*M. angulata*	10887B	33.5	1.811
33	*M. antonii*	12430A	37.5	1.829
34	*M. asterotricha*	10816A	28.0	1.873
35	*M. ayabacana*	10990D	32.0	1.829
36	*M. ayabacana*	10868C	27.0	1.869
37	*M. ayabacana*	11078G	41.5	1.844
38	*M. ayabacana*	11152B	34.0	1.889
39	*M. bennettii*	11581D	4.2	1.804
40	*M. bennettii*	10745A	11.9	1.896
41	*M. bennettii*	10746B	19.5	1.912
42	*M. cuprea*	10744A	8.6	1.890
43	*M. ignea*	11327A	27.0	1.875
44	*M. ignea*	11190B	28.5	1.754
45	*M. klabochorum*	10890A	51.5	1.873
46	*M. leptoura*	11008B	31.5	1.898
47	*M. norae*	11632A	23.5	1.918
48	*M. bicolour*	11598E	24.5	1.853
49	*M. bicolour*	11163B	10.4	1.917
50	*M. bidenta*	11586A	33.0	1.784
51	*M. bonplondi*	10780A	12.1	1.975
52	*M. buccinator*	11600C	10.4	2.070
53	*M. bulbophyllopsis*	10775A	20.5	1.907
54	*M. bulbophyllopsis*	11591C	31.0	1.879
55	*M. cacodes*	11592C	8.5	1.889

Figure 1. **Gel Electrophoresis of PCR reactions amplifying the *matK* locus.** Sample numbers are indicated in the labelling above each lane. Control lanes are indicated with a "-C," as they are negative controls of PCR reactions with water. MW refers to a molecular weight ladder that is labelled at 1000bp. Banding is expected for *matK* around 900bp. (A) Samples 1-13. Sample 2 is weakly banded, but present. (B) Samples 14-23, omitting 15. Sample 15 did not have a high enough extraction for PCR. (C) Samples 24-32. Sample 24 is weakly banded. (D) Samples 33-42. Sample 39 is weakly banded due to procedural error, not all 10uL of PCR reaction went into the well. (E) Samples 43-55 including repeated PCR of sample 24. Samples 48-52, plus samples 24 and 55 are weakly banded but present.

Figure 2. **Phylogenetic trees constructed from sequence alignments of the matK locus of samples 1-23.** (A) Sequences used in alignment are from forward *matK* primers. Tree is constructed from forward primer sequence only. (B) Sequences used in alignments are from reverse *matK* primers. Tree is constructed from reverse primer sequence only. Numbers before OSPF numbers are bootstrap values, and demonstrate the certainty in the location each species is placed in.

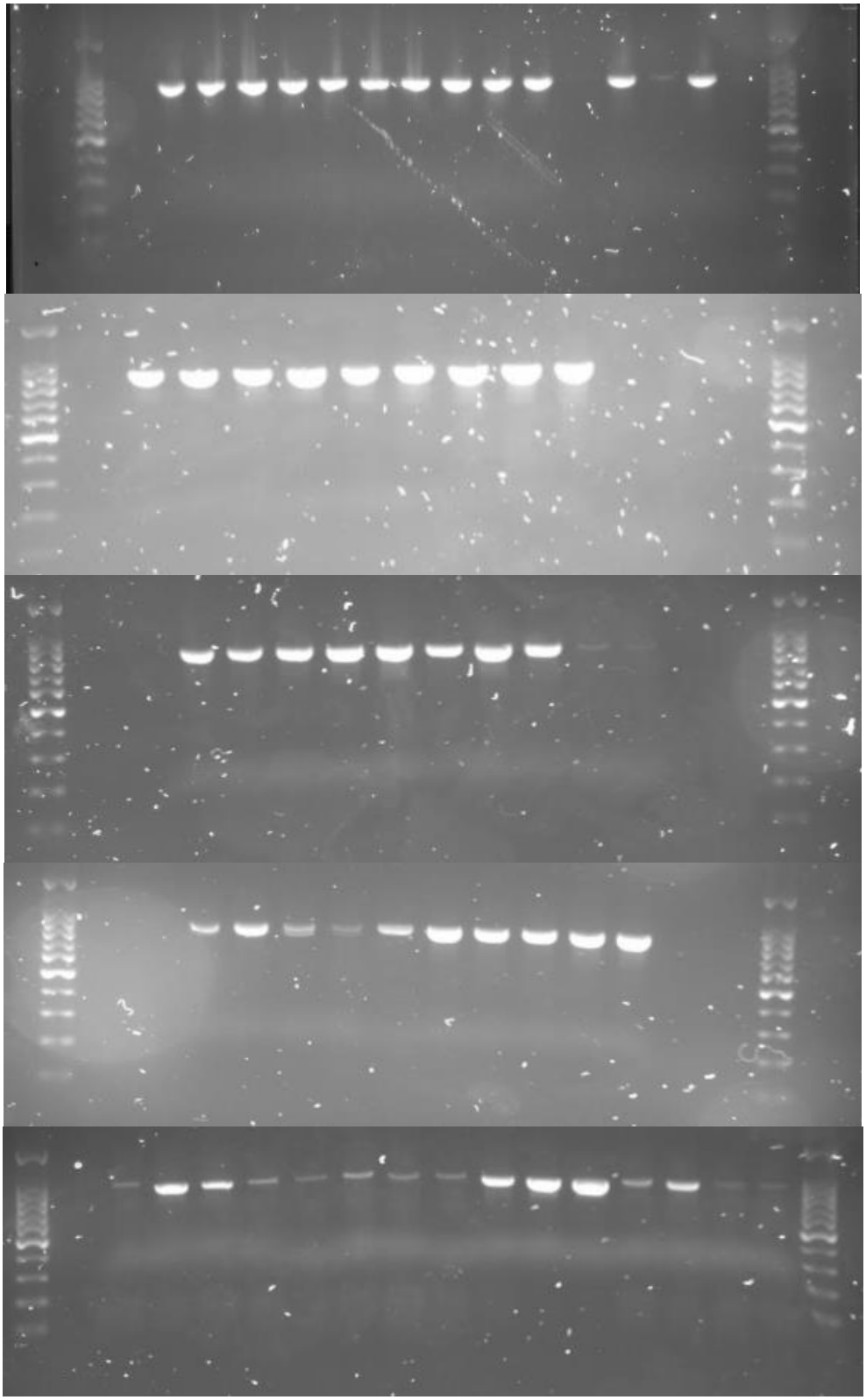

Figure 1: **Gel Electrophoresis of PCR reactions amplifying the *matK* locus.**

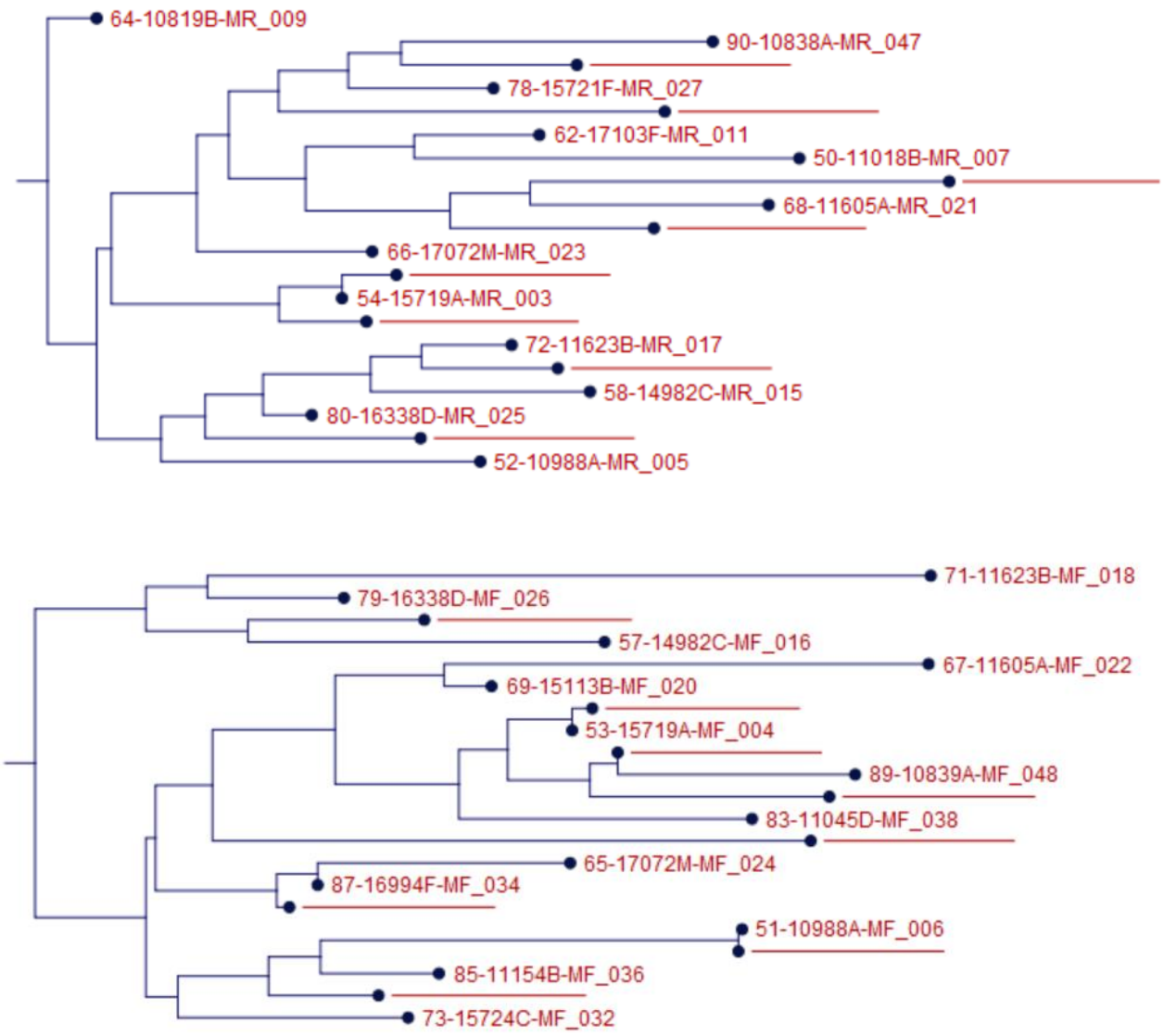

Figure 2. **Phylogenetic trees constructed from sequence alignments of the matK locus of samples 1-23.**