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Short communication

Potential use of DNA barcoding for the identification of tobacco seized from waterpipes

Cindy Carrier, François Cholette *, Camilo Quintero, Chris Fulcher

Alcohol and Tobacco Section, Science and Engineering Directorate, Information, Science and Technology Branch, Canada Border Services Agency, Government of Canada, 79 Bentley Avenue, Ottawa, Ontario K2E 6T7, Canada

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ABSTRACT

DNA barcoding was adopted in our laboratory for the identification of tobacco (*Nicotiana* spp.) in moassel samples seized from "hookah bars". As recommended by the CBOL Plant Working Group, we used a 2-locus combination of rbcL and matK as the plant barcode. As previously reported rbcL routinely produced high quality bi-directional reads but had a lower discriminating power than matK. It was much more difficult obtaining high quality bi-directional reads with matK possibly because of poor sample quality. DNA barcoding successfully identified tobacco in over 60 commercial tobacco moassel products. On the other hand, negative results (no amplification) or the identification of non-tobacco species were obtained from herbal moassel products. Our study clearly demonstrates the practical utility of DNA barcoding beyond taxonomy.

1. Introduction

Chemists who work for the Alcohol and Tobacco section at the Science and Engineering Directorate of the Canada Border Services Agency (CBSA) laboratory analyze samples seized by various government agencies to determine if they contain tobacco. Certificates of analysis are then issued in support of charges under the *Excise Act*, *2001* and other legislations. Determining whether a seized sample contains tobacco is an integral part of the work carried out by the chemists of the Alcohol and Tobacco section.

In recent years, moassel seizures in Canada have been on the rise [1] due to its increasing popularity. Moassel is described as a mixture of tobacco and flavored molasses that is smoked in a waterpipe which is also referred to as narghile, shisha or hookah [2]. Commonly, moassel is smoked in "hookah bars" across many Canadian cities. Unfortunately, the majority of these establishments purchase and sell moassel that has not been properly stamped according to the *Excise Act*, *2001* [3]. Consequently, the appropriate federal and provincial duties are not paid. Moreover, these hookah bars often undermine anti-smoking laws. For example, the Smoke-Free Ontario Act prohibits smoking in public areas such as bars and restaurants [4]. Establishments that allow moassel smoking are subject to substantial fines [4].

To ensure that bars are respecting the Smoke-Free Ontario Act and/or the *Excise Act*, *2001* tobacco enforcement officers will seize products that are suspected to contain tobacco and submit them for analysis. These samples can range from 1 to 5 g of moassel which limits the amount of chemical analysis that can be carried out. As a result, DNA-based approaches were considered since very little plant material is required.

Determining the most appropriate analytical DNA-based method was critical. The CBSA laboratory required an approach that was easy to implement, cost-effective and reproducible. In addition, the method had to be broadly applicable because our laboratory often deals with unknown plant material. DNA barcoding [5] was particularly interesting because (1) it met all of our requirements [6], (2) an official recommendation for a plant barcode was recently made [7], (3) large databases such as GenBank [8] and BOLD [9] are readily accessible, and (4) DNA barcoding can be used successfully to distinguish between various plant species [7]. In this study we demonstrate the potential use of DNA barcoding for the identification of tobacco found in moassel. To the best of our knowledge, this is one of the few rare cases were DNA barcoding has been used in a forensic-type setting.

2. Materials and methods

2.1. Sampling

AC Gayed [10], CT144 [11] and AC Cheng [12] are flue-cured tobacco varieties registered in Canada. These samples were

* Corresponding author. Tel.: +1 613 954 3125; fax: +1 613 952 7825.
E-mail addresses: Cindy.Carrier@cbsa-asfc.gc.ca (C. Carrier), Francois.Cholette@cbsa-asfc.gc.ca (F. Cholette), Camilo.Quintero@cbsa-asfc.gc.ca (C. Quintero), Chris.Fulcher@cbsa-asfc.gc.ca (C. Fulcher).

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Table 1
Description of primers and thermal cycling conditions.[a]

Locus	Primers	Sequence (5′–3′)	Ref.	Thermal cycling conditions
rbcL	rbcLa_R	GTAAAATCAAGTCCACCRCG	[7]	95 °C/10 min followed by 40 cycles of 95 °C/30 s, 55 °C/1 min, 72 °C/1 min
	rbcLa_F	ATGTCACCACAAACAGAGACTAAAGC	[7]	
matK	3F_KIM f	CGTACAGTACTTTTGTGTTTACGAG	[7]	95 °C/10 min followed by 40 cycles of 95 °C/30 s, 52 °C/1 min, 72 °C/1 min
	1R_KIM r	ACCCAGTCCATCTGGAAATCTTGGTTC	[7]	

[a] The minimal amount of DNA required to produce optimal results was determined to be 0.005 ng/μL for *matK* and *rbcL*. For *matK*, a sequence was considered optimal if the overall quality value (QV) [13] was above 20 and ≥750 nt in length. For *rbcL*, a sequence was considered optimal if the overall QV was above 20 and ≥490 nt in length.

obtained from the Canadian Tobacco Research Foundation to be used as reference material/positive controls throughout this study.

Tobacco ($n = 64$) and herbal ($n = 8$) moassel samples were acquired from various government agencies. The samples for this study were selected because they are believed to be a good representation of the brands/flavors commonly distributed in Canada. Samples of various flavors were selected to determine if flavoring compounds have a negative impact on the analysis.

2.2. Sample preparation and DNA extraction

Approximately 100 mg of moassel was frozen in liquid nitrogen for a minimum of 30 s. Immediately afterwards, the plant material was pulverized for 30 s at 30 Hz using a Retch universal mixer-mill disrupter model number MM301 (Thermo Fisher Scientific, Ottawa, Canada). DNA extractions from pulverized moassel were carried out automatically in a QIAcube (Qiagen, Mississauga, Canada) using the DNeasy plant mini kit (Qiagen). The DNA concentrations and the quality (A260/A280) of the extracts were estimated with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). All DNA extracts were stored at −20 °C until analysis.

2.3. PCR

Primers for the *rbcL* and *matK* loci (Table 1) were supplied by Applied Biosystems (Foster City, CA). A 25 μL reaction contained 12.5 μL of AmpliTaq Gold 360 master mix (Applied Biosystems), 3.75 μL (2.0 μM) of each primer and 5.0 μL of DNA extract. The amplifications were carried out in a Veriti thermal cycler (Applied Biosystems) using conditions detailed in Table 1. Amplification checks were carried out in an Experion automated electrophoresis station (Bio Rad, Mississauga, Canada) using the Experion DNA 1 K analysis kit (Bio Rad). Amplicons were then purified automatically in a QIAcube (Qiagen) using the QIAquick PCR purification kit (Qiagen). To determine how much DNA template was required to produce optimal results for *rbcL* and *matK* we prepared six serial diluted concentrations (1.5, 0.5, 0.05, 0.005, $5E^{-4}$ and $5E^{-4}$ ng/μL) of CT144 DNA. These serial dilutions were prepared in triplicate.

2.4. Cycle sequencing

Bi-directional cycle sequencing was carried out with the primers listed in Table 1 with the BigDye v3.1 cycle sequencing kit (Applied Biosystems). All cycle sequencing reactions were carried out in a Veriti thermal cycler (Applied Biosystems). Cycle sequencing reactions were purified with the help of the BigDye XTerminator kit (Applied Biosystems) prior to sequence analysis. Bi-directional sequences were determined using a 3500 Genetic Analyzer (Applied Biosystems).

2.5. Sequence analysis and identification

Contig sequences were assembled with the help of the DNASTAR software suite version 2.0.0.78 (DNASTAR, Madison, WI). Samples were identified by using BLASTn [14] in GenBank and the Plant Identification search engine in BOLD [9].

3. Results and discussion

No attempts were made to separate plant material from the molasses during DNA sample preparation. The goal was to keep the protocol as stream-lined as possible while minimizing the risk of cross-contamination. On average, 0.7 μg (±0.4 μg) of DNA was extracted from approximately 100 mg (±5 mg) of moassel. In order to approximate the quality of the extracts, we relied on the A260/A280 values. These values varied greatly, ranging from 0.99 to 2.07. It is suspected, that the large variability in these values was caused by flavoring compounds, found in the molasses, which elute along with DNA. Regardless of low-quality extracts, the PCR amplifications remained unaffected (Fig. 1). This demonstrates that readily amplifiable DNA can be extracted from moassel in its usual form using the DNeasy plant mini kit.

The *rbcL* fragment was successfully amplified (approximately 620 bp) from tobacco moassel regardless of flavor (Table 2, Fig. 1). Likewise, the *matK* fragment (approximately 800 bp) amplified with all tobacco moassel also (Table 2, Fig. 1). On the other hand, the amplification of the *rbcL* and *matK* loci was far less successful with herbal moassel. Out of eight herbal moassel samples, amplification was only successful with three samples (Table 2, Fig. 1). Furthermore, only the *rbcL* fragment could be amplified. The low success rate of amplification for these samples was likely caused by manufacturing practices rather than flavoring compounds. Anecdotal reports from the field suggest that bagasse, a by-product of the cane sugar industry [15], is the primary ingredient of herbal moassel. If so, it is possible that bagasse processing causes DNA degradation [16] to a point were it is unusable for DNA barcoding. Unfortunately, it is not possible to confirm if bagasse is being used as base material for herbal moassel because manufacturers are hesitant to divulge the contents of their products.

High quality bi-directional sequences were routinely obtained from *rbcL* as previously reported [7]. Contig sequences of 490 nt or higher were regularly attained from tobacco moassel (Table 2). High quality contigs were also achieved with herbal moassel when amplification was successful. In the majority of cases, manual editing of the contig sequences was not required at all. Obtaining high quality bi-directional reads from *matK* was problematic, which was somewhat expected [7]. Contig sequences of the expected size (around 800 nt) were difficult to achieve consistently with tobacco moassel (Table 2).

BLASTn searches of the *rbcL* and *matK* contigs in GenBank produced similar results (Table 2). In both cases, the best results were a variety of *Nicotiana* species. However, resolution significantly improved when the *matK* contigs were 800 nt and above. If amplification was successful with herbal moassel, the plant material was identified as *Triticum aestivum* (common wheat). Likewise, the top matches (for tobacco based samples) in BOLD with *rbcL* sequences were a mixture of *Nicotiana* species (Table 2). On the other hand, *matK* contigs provided species level identifications

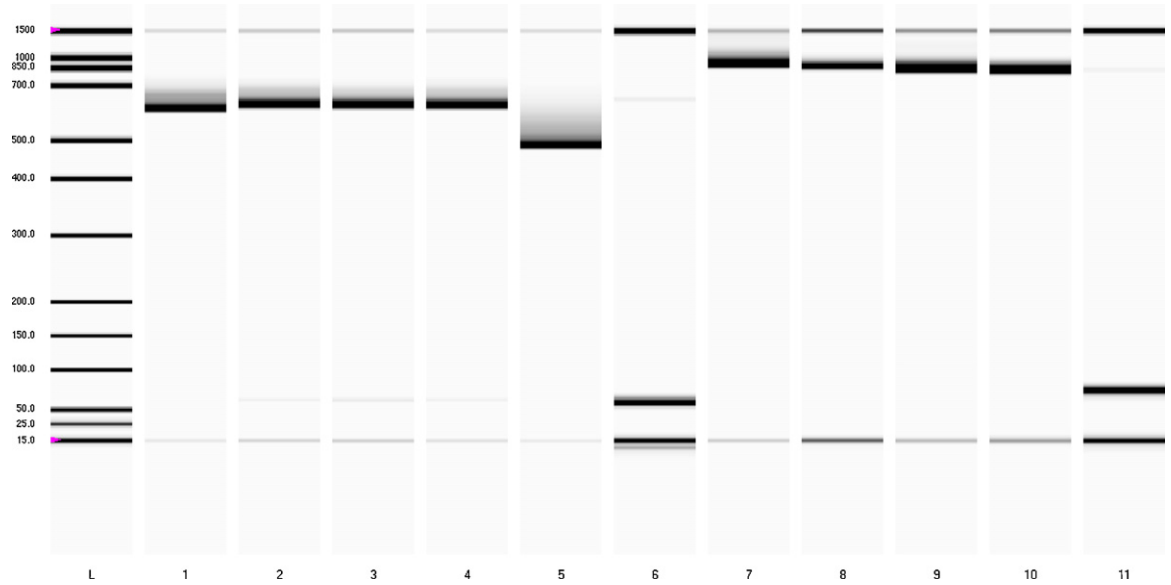

Fig. 1. Typical virtual gel of amplicons generated by the *rbcL* and *matK* primers. Lane L, DNA 1 K ladder; lane 1, amplification of *rbcL* from reference tobacco; lanes 2–4, amplification of *rbcL* from tobacco moassel (~620 bp) of various flavors; lane 5, successful amplification of *rbcL* from herbal moassel (~500 bp); Lane 6, unsuccessful amplification of *rbcL* from herbal moassel; lane 7, amplification of *matK* from reference tobacco; lanes 8–10, amplification of *matK* from tobacco moassel (~800 bp) of various flavors; and lane 11, unsuccessful amplification of *matK* from herbal moassel. The Experion DNA 1 K analysis kit relies on two internal markers to normalize the migration times among samples. These markers are visible at 1500 bp (upper marker) and 50 bp (lower marker).

Table 2
Snapshot of barcoding results.

Flavor or variety	Moassel type	Identification							
		GenBank				BOLD			
		rbcL	Sequence length (nt)	*matK*	Sequence length (nt)	*rbcL*	Sequence length (nt)	*matK*	Sequence length (nt)
AC Cheng	n/a	*Nicotiana* spp.[a]	≥540	*Nicotiana spp.*[c]	>800	*Nicotiana* spp.[e]	≥540	*Nicotiana tabacum*	>800
AC Gayed	n/a	*Nicotiana* spp.[a]	≥540	*Nicotiana spp.*[c]	>800	*Nicotiana* spp.[e]	≥540	*Nicotiana tabacum*	>800
CT144	n/a	*Nicotiana* spp.[a]	≥540	*Nicotiana spp.*[c]	>800	*Nicotiana* spp.[e]	≥540	*Nicotiana tabacum*	>800
Apple	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[c]	>800	*Nicotiana* spp.[f]	≥490	*Nicotiana tabacum*	>800
Melon	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[d]	750–800	*Nicotiana* spp.[f]	≥490	*Nicotiana spp.*[g]	750–800
Peach	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[d]	750–800	*Nicotiana* spp.[f]	≥490	*Nicotiana spp.*[g]	750–800
Apricot	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[d]	750–800	*Nicotiana* spp.[f]	≥490	*Nicotiana spp.*[g]	750–800
Grape/Mint	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[c]	>800	*Nicotiana* spp.[f]	≥490	*Nicotiana tabacum*	>800
Grenadine	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[c]	>800	*Nicotiana* spp.[f]	≥490	*Nicotiana tabacum*	>800
Vanilla	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[d]	750–800	*Nicotiana* spp.[f]	≥490	*Nicotiana spp.*[g]	750–800
Jasmine	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[c]	>800	*Nicotiana* spp.[f]	≥490	*Nicotiana tabacum*	>800
Liquorice	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[c]	>800	*Nicotiana* spp.[f]	≥490	*Nicotiana tabacum*	>800
Cappuccino	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[c]	>800	*Nicotiana* spp.[f]	≥490	*Nicotiana tabacum*	>800
Lemon	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[c]	>800	*Nicotiana* spp.[f]	≥490	*Nicotiana tabacum*	>800
Pistachio	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[c]	>800	*Nicotiana* spp.[f]	≥490	*Nicotiana tabacum*	>800
Guava	Tobacco	*Nicotiana* spp.[a]	≥490	*Nicotiana spp.*[d]	750–800	*Nicotiana* spp.[f]	≥490	*Nicotiana spp.*[g]	750–800
Apple	Herbal	–[b]	–	–	–	–	–	–	–
Aniseed	Herbal	–	–	–	–	–	–	–	–
Coffee	Herbal	–	–	–	–	–	–	–	–
Cocktail	Herbal	*Triticum aestivum*	≥500	–	–	*Triticum aestivum*	≥500	–	–
Grape	Herbal	–	–	–	–	–	–	–	–
Orange ($n=2$)	Herbal	*Triticum aestivum*	≥500	–	–	*Triticum aestivum*	≥500	–	–
Watermelon	Herbal	–	–	–	–	–		–	–

[a] Top BLASTn results in GenBank with the *rbcL* sequence: *N. glauca*, *N. petunioides*, *N. noctiflora*, *N. sylvestris*, *N. tabacum*, *N. debneyi* and *N. acuminata*. In each case results were identical (scores, E-values, identities, etc.).
[b] No amplification.
[c] Top BLASTn results in GenBank with *matK* sequence: *N. tabacum*, *N. sylvestris*, and *N. digluta*. In each case results were identical (scores, E-values, identities, etc.).
[d] Top BLASTn results in GenBank with *matK* sequence: *N. goodspeedii*, *N. occidentalis*, *N. umbratica* and *N. benthamiana*. In each case results were identical (scores, E-values, identities, etc.).
[e] Top Plant Identification results in BOLD Systems with the *rbcL* sequence: *N. debneyi*, *N. tabacum*, *N. petunioides* and *N. noctiflora*. In each case results were identical (score, similarity and E-value).
[f] Top Plant Identification results in BOLD Systems with the *rbcL* sequence: *N. noctiflora*, *N. tabacum*, *N. petunioides*, *N. debneyi* and *N. glauca*. In each case results were identical (score, similarity and E-value).
[g] Top Plant Identification results in BOLD Systems with the *matK* sequence: *N. benthamiana*, *N. goodspeedii*, *N. occidentalis* and *N. umbratica*. In each case results were identical (score, similarity and E-value).

(*Nicotiana tabacum*, i.e. cultivated tobacco) if the sequences were above 800 nt in length. If the *matK* sequences were below 800 nt, the proper genus was still identified. Although identification at the species level was not always possible, we are confident that the correct genus was assigned [17]. Since all species of the *Nicotiana* genus [18,19] are considered "tobacco", DNA barcoding would be well-suited for the identification of tobacco moassel. According to our results, DNA barcoding would provide enough discrimination to determine if a sample could be considered a tobacco product and require proper stamping and payment of excise duty under the *Excise Act*, *2001*.

During our study we only assayed the core plant barcode recommended by the CBOL Working group [7]. However if identification at the species level would have been critical alternative barcoding regions would have been considered. Recently the China Plant BOL Group recommended that the internal transcribed spacer (ITS) be incorporated into the core plant barcode because it shows a high level of discriminatory power [20]. The ITS could be potentially useful in a forensic laboratory because this marker can be amplified into two shorter subunits (ITS-1 and ITS-2) [21]. Quite often, in a forensic setting, samples have severely degraded DNA therefore the amplification of a full length barcode may not always be possible. The *trnH-psbA* spacer might also have been a barcoding region of interest. This spacer showed a high sequence divergence and amplification success when comparing *N. tabacum*, *Atropa belladonna*, and significantly divergent angiosperms [22]. Also, the *trnH-psbA* spacer is attractive due to its short size (about 450 bp in *N. tabacum*) [22]. As a result, it could be amendable to degraded DNA.

Recently, DNA barcoding was applied to a case where unknown plant material, declared as "almond leaves", was being imported into Canada from Haiti. Border Service Officers seized the unknown plant material suspecting that it might be tobacco. DNA barcoding revealed that the plant material was from the *Terminalia* genus. This genus of large trees includes species like *T. catappa*, which is used in traditional Haitian medicine [23]. Chemical analysis also suggested that the plant material in question was not tobacco further supporting barcoding results. This clearly demonstrates the practical utility of DNA barcoding for these kinds of samples.

4. Conclusions

This study shows that DNA barcoding has some practical applications beyond taxonomy, including the identification of tobacco in moassel. Even though species identification was not always possible, identification at the genus level is adequate for the needs of the CBSA laboratory. DNA barcoding proved to be especially useful when sample size was limited. In the future, other primer sets will be assayed to improve the identification of plant material in herbal moassel.

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