DNA Mini-Barcodes to Help Identify Species of the Family Ranunculaceae

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Abstract

Some members of the plant family Ranunculaceae are used as **spices** or **medicinal plants**. Other members are the opposite and can be **irritant** or even **poisonous**. Therefore, it is very important to correctly identify the species present in botanically derived samples, viz., dietary supplements, or other finished products, to ensure authenticity and safety.

Species identification with universal primers is relatively simple, however, these molecular markers have limited utility for multi-herbals or herbal products with exogenous components. As a part of our ongoing pursuit to develop species-specific DNA mini barcodes, a diagnostic PCR product has been developed using six designed primers that bind specifically to Ranunculaceae. The resulting PCR product with a ~200 bp mini barcode was validated with at least nine additional Ranunculaceae plant samples. Applicability of such tools would be helpful for the authenticity, species specificity of various Ranunculaceae-based finished products often known to possess only damaged/fragmented DNA.

Introduction

The identification of plant material using DNA barcodes and other genomic regions useful for species identification is becoming more and more a standard procedure. In the past, **universal primers**, eg. ITS1/ITS4 have been used to amplify diagnostic genomic regions from plants. These primers are unspecific as they preferably amplify from the majority of plant (or fungal) material present and would most likely not satisfactorily amplify from material that may be present in a low percentage. We present here primers that are **less universal and more family-specific,** that allow the amplification of short diagnostic DNA regions of about 150-200 bp in length, DNA mini barcodes, from a variety of genera from the family Ranunculacea Juss..

The members of the family Ranunculaceae contain the **toxic compound** protoanemonin (aka. anemonol or ranunculol). The toxicity of Ranunculaceae species seems to be more an issue for livestock that accidentally ingest it than for humans. Various Ranunculaceae species have economic importance as ornamentals from the genera *Ranunculus* L., *Delphinium* L., *Naravelia* L., *Clematis* L., *Nigella* L. and *Caltha* L.. Medicinally used species are from the genera *Aconitum* L., *Clematis* L., *Cimicifuga* Wernisch., *Helleborus* L., *Caulophyllum* Michx., and *Hydrastis* L.. The seeds of *Nigella sativa* L. are used as **spice** in pickles.

Identification of Ranunculaceae species by DNA has focused mainly on population studies, analyzing divergences among (few) species per genus. The development of species-specific primer sets based on a single genomic region is often time-consuming and sometimes not possible due to the lack of sufficient differences in the nucleotide sequence to make primers species-specific. Also, by using species-specific primers undesired non-targeted related species may go undetected.

The designed primers, based on the ITS genomic region, in this study result in DNA mini barcodes (approximately 90-200 bp long) suitable to amplify from species of a variety of genera that, after sequencing, can be helpful to identify the species it was amplified from. The presented primers were suitable for samples of the genera *Aconitum*, *Clematis*, *Cimicifuga*, *Helleborus*, *Caulophyllum*, *Nigella*, and *Hydrastis*. These mini barcodes were used to detect and identify plant material present in a variety of **dietary supplements** (raw plant material, watery solutions, oil or capsules) and **homeopathic medicines** (pellets).

Materials and Methods

The sample material was kindly donated by the Pullen Herbarium, University of Mississippi (Oxford, MS) and the Missouri Botanical Garden (St Louis, MO). Additional samples were provided and authenticated by Dr. Aruna D. Weerasooriya or Dr. Vijayasankar Raman, National Center for Natural Product Research (NCNPR), University of Mississippi (Table 1, 2).

Genomic DNA extractions were performed using the DNEasy mini kit (Qiagen) with small modifications. In brief: to either 50-100 mg raw plant material, or the either visible or non-visible pellet of 10 ml of watery solutions or oil, 20-50 mg Polyvinylpolypyrrolidone (PVPP), two stainless metal balls were ground to a fine powder using a mixer mill MM 400 (Retsch). After the sample was ground, twice the amount of buffer AP1 for genomic DNA extraction from raw plant material were added and then handled according to the manufacturer's instructions.

To ten homeopathic pellets 10-15 mg PVPP were added and dissolved in 1,600µL buffer AP1 and 8 µL of RNAse and incubated at 65°C for 1 hr. The sample was centrifuge for 1 min at 20,000 x g and 800 µL of the supernatant was discarded. The remaining approximately 800 µL were then handled according to the manufacturer's instructions.

Table 1. Analyzed raw plant material samples

Sample ID	Genus	Species
12394	*Ranunculus*	*abortivus* L.
12419	*Ranunculus*	*abortivus* L.
14686 (colored sol.)	*Ranunculus*	*acris* L.
14263	*Ranunculus*	*aquatilis* L.
14568	*Ranunculus*	*repens* L.
10596	*Ranunculus*	*sceleratus* L.
15519	*Ranunculus*	*septentrionalis* Poir.
2033	*Aconitum*	*napellus* L.
9299	*Actaea*	*americana* D.C.
3599	*Actaea*	*racemosa* L.
3609 (colored sol.)	*Actaea*	*racemosa* L.
1944	*Adonis*	*vernalis* L.
14492	*Caltha*	*palustris* L.
401	*Clematis*	*aethusifolia* Turcz.
6547	*Clematis*	*pitcheri* Torr. & A.Gray
2602	*Delphinium*	*andersonii* A.Gray
14321	*Helleborus*	*caucasicus* A. Braun
2067	*Helleborus*	*niger* L.
3634	*Hydrastis*	*canadensis* L.
2352	*Hydrastis*	*canadensis* L.
5888	*Nigella*	*sativa* L.
2924	*Nigella*	*sativa* L.
14440	*Thalictrum*	*foetidum* L.

Primers were designed to be **less universal and more family-specific,** also 5' tailed with NexteraXT **adapter sequences** to facilitate a second PCR with only one set of primers. PCR amplifications were carried out in two consecutive reactions. The first PCR consist of a 25 µL reaction mixture containing 2 µL of a DNA solution (undiluted, 1:10, 1:100), PCR mix and Ranunculaceae primers as multiplex. The first PCR was a 10 cycle **touchdown** program with annealing temperatures of 68°C to 53°C. The second PCR consisted of a 25 µL reaction mixture using 1 µL of the first PCR as template and NexteraXT- Forward/Reverse primers. The second PCR program was a classic PCR of 30 cycles. After amplification, an aliquot was analyzed by electrophoresis on a 1.5% borate agarose gel, visualized under UV light. Successfully amplified PCR products were isolated and **sequenced** in both directions at GeneWiz. Sequences were analyzed with DNASTAR and Clone manager 9 (Scientific & Educational Software) software. Contig sequences were screened against the NCBI nucleotide Database BLAST.

Results and Discussion

Primers were successfully designed for the ITS region to amplify a mini-barcode from several Ranunculaceae genera (Table 1). Due to the degeneracy of the designed primers resulting in TMs (melting temperature) ranging 56°C-70° a **two-step PCR** was carried out.

Twenty-one out of 23 samples resulted in a PCR product when the Ranunculaceae primers were used for amplification on a variety of raw plant material from Ranunculaceae genera (Table 1). Detected products contain the 67 bp NexteraXT- adapter sequences and appear therefore at a size of approximately 200-250 bp (Fig. 1). Sequencing of the PCR products confirmed sample identity.

The DNA solution of samples 14686 and 3609 was slightly colored indicating that the solution contained phenolic compounds that are known to interfere with PCR, which could be the reason why no amplification was detected from these two DNA solutions.

All of the sixteen dietary supplements and homeopathic medicines analyzed eventually resulted in a PCR product, some only when template was undiluted DNA (e.g. DSRa03), some only when template was a 1:100 dilution (e.g. DSRa07) (Fig. 2).

The designed primers presented here were tested for their ability to detect Ranunculaceae **DNA present in a mix of eleven non-Ranunculaceae DNAs.** Twenty-one out of 23 samples resulted in the characteristic single PCR product when the Ranunculaceae primers were used for amplification. This shows the primers are able to detect Ranunculaceae DNA when present in a DNA mix and therefore may be helpful to detect adulterating Ranunculaceae plant material. The detection limits were not determined.

Table 2. List of analyzed dietary supplements, homeopathic medicines, and derived PCR products

ID	Name on label	Label information	undil.	1:10	1:100
DSRa01	Aconitum napellus	12C pellets, homeopathic medicine	+	-	-
DSRa02	Aconitum napellus	Watery solution	+	-	-
DSRa03	Aconitum napellus	6C, pellets, homeopathic medicine	+	-	-
DSRa04	Black seed (Nigella)	Capsules	+	+	+
DSRa05	Black seed (Nigella)	Oil - cold pressed	+	+	+
DSRa06	Black Cohosh	Raw plant material in capsule, (root, root extract)	-	+	+
DSRa07	Black Cohosh	Raw plant material in capsule, (root)	-	-	+
DSRa08	Blue Cohosh	Bulk Herbs, root (wild harvested)	+	+	+
DSRa09	Blue Cohosh	Raw herb	+	+	+
DSRa10	Blue Cohosh	Root, capsules	-	+	+
DSRa11	Clematis	Chinese Clematis powder (root & rhizome)	+	-	-
DSRa12	Clematis	Clematis Powder	+	-	-
DSRa13	Helleborus niger	200C pellets, homeopathic medicine	+	-	-
DSRa14	Helleborus niger	6C pellets, homeopathic medicine	+	-	-
DSRa15	Hydrastis canadensis	6C pellets, homeopathic medicine	+	-	-
DSRa16	Nigella sativa	Oil	+	-	+

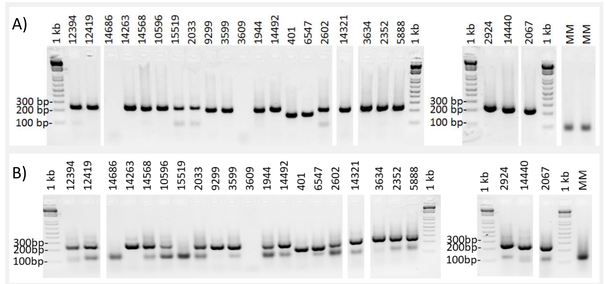

Figure 1. Agarose gel image of PCR products derived from applying the Ranunculaceae primer mix. Template was DNA from various Ranunculaceae genera samples. Lack of detectable PCR product from samples 14686 and 3609 was most likely due to compounds in the DNA solution interfering with PCR. A) Raw plant material. B) PCR mix with eleven non-Ranunculaceae DNA. MM = master mix (no DNA template), 1kb = molecular size standard 1kb plus DNA ladder

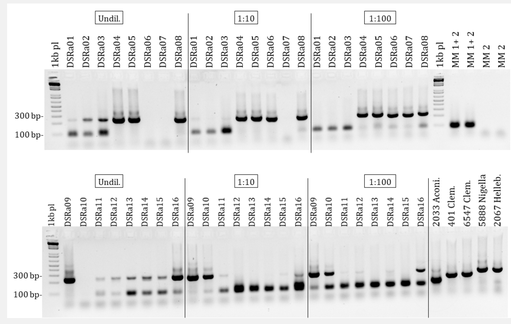

Figure 2. Agarose gel image of PCR products applying the Ranunculaceae primer mix. Template was DNA isolated from various dietary supplements and homeopathic medicines. 'DSRa' (Table 1, Table 2). Positive controls (2033, 401, 6547, 2067). Template DNA solution was either undiluted, 1:10, or 1:100. Negative controls were master mix without any DNA added. All of the sixteen samples analyzed eventually resulted in a PCR product, some only when template was undiluted DNA (e.g. DSRa03), some only when template was a 1:100 dilution (e.g. DSRa07). The intense band at approximately 100 bp are unincorporated primers. MM = master mix (no DNA template), 1kb = molecular size standard 1kb plus DNA ladder

Conclusion

The overall goal is to design primers that can amplify DNA mini-barcodes to identify plant origins even of processed material e.g. present in dietary supplements. The design of **less universal and more family-specific primers** presented here also falls into the **mini-barcode** category. They not only allow the **amplification from damaged/degraded DNA** but also the detection of target DNA when present in a mix of DNA. In addition the primer adapter sequences would allow the analysis by High-Throughput-Sequencing, which is the more preferred method for DNA barcoding over Sanger sequencing.

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