A Streamlined Method for Identifying Foraging Targets of Native Bees Using Multiple-Loci DNA Barcoding

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Introduction

- The pairing of local plant and bee species could allow selective plantings to promote the regional native bee community, or even target those that pollinate specific crops (Williams *et al.*, 2015; Blaauw and Isaacs, 2014).
- Previous research has demonstrated the feasibility of identifying plant-pollinator interactions using pollen DNA metabarcoding (Sickel *et al.*, 2015; Bell *et al.*, 2017). However, this type of high-throughput method requires costly equipment and cannot quantify proportions of taxa per pollen load.
- Amplifying multiple loci from a single pollen grain provides a simple, lower cost method for defining plant-pollinator interactions and can be performed in a basic laboratory, such as those found in an undergraduate or community college setting.
- Further more, the amplification and sequencing of two or more DNA loci is thought to increase discriminatory power (Kress and Erickson, 2007).
- Therefore, the goal of the present work is to develop a simple method for identifying native bee foraging habits using a three-loci, PCR, DNA-based identification of single pollen grains taken from sampled native bees.

Methods

Table 1. List of primers used for amplification and sequencing of three DNA regions from unknown single pollen grains.

Primer Set	Primer Name	Binding	Primer Sequence (5'-3')	Reference
rbcL	1f	Forward	ATGTCACCACAAACAGAAACTAAAGC	Käss and Wink, 1997
	724r	Reverse	TATCCATTGCTGGGAATTCAAATTTG	
trnH-psbA	Fwd PA	Forward	GTTATGCATGAACGTAATGCTC	Kress and Erickson, 2007
	Rev TH	Reverse	CGCGCATGGTGGATTCACAATCC	
ITS2	S2F	Forward	ATGCGATACTTGGTGTGAAT	Chen *et al.*, 2010
	S3R	Reverse	GACGCTTCTCCAGACTACAAT	

- Bees of the genus *Bombus* were caught via aerial net and vortexed singularly in 15mL of nuclease-free water to suspend the total pollen load and facilitate the random sampling of pollen grains. Pollen removal from bees was confirmed visually under a stereomicroscope.
- 5µL of the pollen suspension was pipetted on to a microscope slide and allowed to dry. The pollen was stained with 10µL of an aqueous solution containing 0.7 mM aniline blue and 30mM sucrose, then spread thinly across the slide to separate and count grains. Stained pollen grains were identified as viable.
- Single viable pollen grains were isolated under a stereomicroscope and transferred to PCR tubes containing one (single), two (duplex), or three (multiplex) sets of primers (Table 1), Phire™ Plant Direct PCR Master Mix, and nuclease-free water. Positive control experiments were performed using genomic pea DNA (*Pisum sativum*).
- 25µL PCR amplifications were performed for 35 cycles, at an annealing temperature of 60˚C, and an extension time of 30 sec, as outlined by the Phire™ Plant Direct PCR protocol.
- PCR products were analyzed using 0.8% agarose gel electrophoresis and the Gel Doc™ EZ Imager.
- To confirm and separate the products of the multiplex reaction, a second round of PCR amplifications were carried out in three separate tubes using 0.5µL of the multiplex product as template and a different primer set for each tube.
- Products from all three amplifications, originating from the same pollen source, were purified and sequenced using an ABI genetic analyzer.
- Sequencing results were analyzed using the nucleotide Basic Local Alignment Search Tool (BLASTn) database to identify the plant species from which the single unknown pollen grain originated.

Results

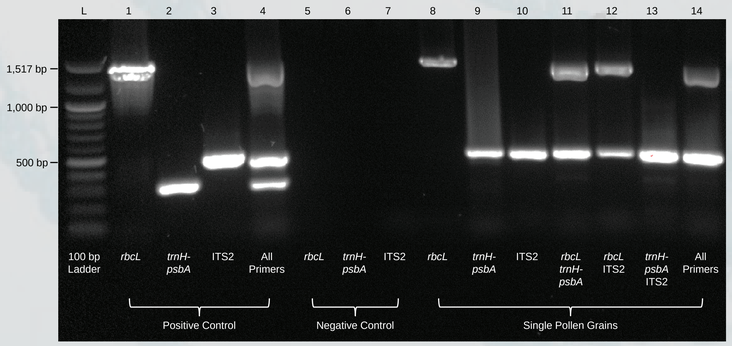

Figure 1. 0.8% agarose gel electrophoresis of PCR products. Results show amplification of genomic pea DNA (lanes 1-4) and seven unidentified single pollen grains (lanes 8-14) using primers *rbcL*, *trnH-psbA*, and ITS2 for single, duplex, and multiplex reactions.

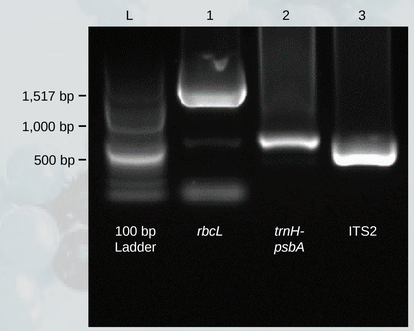

Figure 2. 0.8% agarose gel electrophoresis of PCR products. Results show amplification of DNA templates (lanes 1-3) obtained from the products of the multiplex reaction (fig. 1, lane 14) using primers *rbcL*, *trnH-psbA*, and ITS2, each in their own separate reaction. This confirms successful amplification of the three loci in a multiplex reaction.

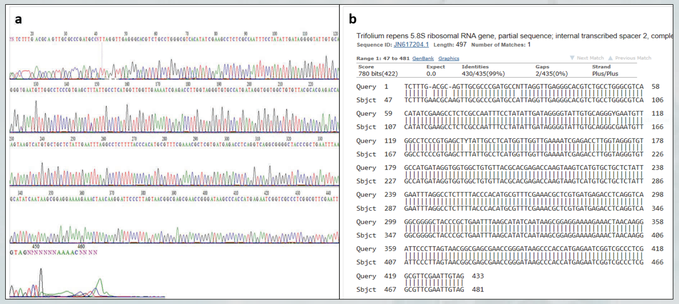

Figure 3. Chromatogram (a) and sequence alignment from the BLASTn database (b) of the ITS2 region obtained from the unknown pollen grain sampled for the multiplex reaction. The sequence confirms that the unknown pollen grain is 99% similar to *Trifolium repens* (white clover).

Discussion

- Amplification of multiple loci using both genomic pea DNA and single unknown pollen grains sampled from *Bombus* were successful for all three loci. However, *trnH-psbA* and ITS2 displayed better amplification than *rbcL* in duplex and multiplex reactions (Fig. 1).
- The *trnH-psbA* amplicon for the control pea DNA was found to be smaller (~300 bp) than the products obtained from the unknown pollen grains (~480 bp) (Fig. 1). This is due to the difference in size of their intergenic spacer regions (Fig. 4), which is consistent with previous work that reports the amplicon size of *trnH-psbA* to vary from plant to plant (Kress *et al.*, 2005).
- As a result, DNA bands for *trnH-psbA* and ITS2 from unknown pollen grains migrated together on agarose gel due to their similar sizes (Fig. 1). This explains the missing bands when both primer sets were used in duplex and multiplex reactions (Fig. 1, lanes 13 and 14).
- Despite the co-migration of the *trnH-psbA* and ITS2 amplicons, the second round of PCR amplifications using template DNA from the multiplex reaction confirmed the presence of amplicons for all three loci in the multiplex PCR tube (Fig 2).
- Sequencing results and Blastn database analyses of the three amplicons from the multiplex PCR revealed the unknown pollen grain to originate from *Trifolium repens*, commonly known as white clover (Fig 3). This confirms that single unknown pollen grains from native bees can be successfully identified using multi-loci DNA barcoding.

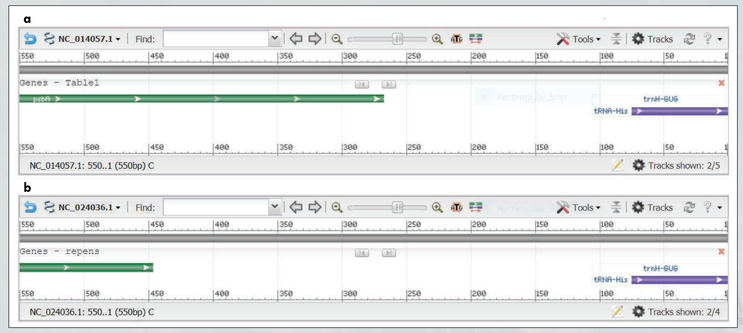

Figure 4. Browser view from the BLASTn database depicting the difference in size of the intergenic spacer region between *trnH* and *psbA* for *Pisum sativum* (pea) (a) and *Trifolium repens* (white clover) (b).

Conclusion

- The current work shows that multiple DNA loci from a single pollen grain can be amplified using multiple primer sets in a single multiplex reaction.
- This approach ensures high discriminatory power for the species identification of pollen grains collected by native bees.
- This method can be performed with basic lab equipment, minimal training and little expense, making it ideal for a teaching experience that produces valuable data for future studies.

Acknowledgements

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