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NOTE

Identification of leaf-mining insects via DNA recovered from empty mines

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Abstract

We report the development of an improved method for the extraction and amplification of leaf miner DNA recovered from empty mines. Our method is simple, easy to use, and foregoes the time-consuming task of scraping out mines required by previous methods. We collected leaves with 1- and 2-day-old vacated mines, cut out and then ground the mined portions, and amplified the mtDNA COI barcode sequence using universal insect primers. We obtained high-quality sequences for 31% of our empty mines: 20% yielded sequences associated with a leaf miner species; and an additional 11% yielded sequences associated with whiteflies, mites, or fungi. Our improved method will facilitate ecological studies determining herbivore community dynamics and agricultural studies for pest monitoring and identification.

Key words: herbivory, barcode, molecular

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Introduction

Leaf miners are a diverse group of herbivores, with approximately 10 000 species worldwide belonging to at least four insect orders (Coleoptera, Diptera, Hymenoptera, and Lepidoptera; Auerbach et al. 1995). In many natural systems, leaf miners are highly prevalent and can have substantial impacts on their hosts (Raimondo et al. 2003). Furthermore, leaf miners can be major pests in agricultural and horticultural systems, reducing crop production and esthetic value (Hering 1951; Parella and Keil 1984; Raimondo et al. 2003). Some leaf-mining pests are invasive; of these, some represent entirely new taxa for an area, whereas others displace native congeners that they resemble closely (Abe and Tokumara 2008). Thus, accurate identification of leaf-mining species is extremely important, both in natural systems and for the monitoring of actually or potentially invasive species.

However, for a number of reasons, leaf miners can be very difficult to identify. Adults do not associate with their host plants until oviposition; hence, adult collections cannot typically be connected to host plant species. Larvae develop inside leaves (feeding on the parenchyma cells; Connor and Taverner 1997), where they can be inconspicuous for most of their life cycle. Most leaf mines are detected late (i.e., once the mine has become apparent), but by then the larva might have reached its last instar or vacated the mine as an adult. Even if larvae have been collected, they are difficult to identify because they have few easily scored morphological characters (Spencer 1990). Adults can be morphologically identified, but it is logistically difficult to rear enough individuals to obtain large sample sizes, and identification can still be challenging for non-experts because of the difficulty of locating and using identification tools (Derocles et al. 2015). Finally, generalist miners (based on adult morphology)

may prove to be complexes of cryptic host races (e.g., the holly leaf miner *Phytomyza glabricola* (Diptera: Agromyzidae; Scheffer and Hawthorne 2007) or cryptic species (J. Mlynarek, personal observation, 2015). These difficulties suggest great value for techniques that could identify leaf miners without depending on morphological characters, especially for mines that have already been vacated.

Molecular techniques for species identification are constantly improving. Sequence-based identification of specimens in-hand is now routine (Joly et al. 2013). Holometabolous insects can be identified by barcoding empty puparia (Mazzanti et al. 2010), and host insects from parasitoids (including leaf miners) can be identified using DNA sequencing from post-emergence pupa or puparia (Hrcek et al. 2011; Condon et al. 2014). Environmental DNA can even be used to simultaneously identify entire communities (e.g., Taberlet et al. 2012). Recently, Derocles et al. (2015) developed a method to amplify DNA from empty leaf mines. They opened mines and scraped the inside of the mine to collect larval skins and frass (which may include sloughed gut cells), and sequenced either the insect barcode (a 658 bp fragment of COI) or a mini-barcode (a 130 bp fragment of COI) from the scrapings. However, their method is laborious, and they had a low success rate of amplification (6%) for older mines.

We have developed an alternative method for the extraction and amplification of leaf miner DNA from empty mines. Our method is simple and foregoes the time-consuming task of scraping out mines of Derocles et al. (2015) with greater sequencing success.

Methods

We collected 96 mined leaves of *Doellingeria umbellata* (Asteraceae) from the University of New Brunswick Woodlot (Fredericton, New Brunswick, Canada; 45.915, −66.648) in July 2015. We chose leaves with "blotch" mines (**Fig. 1**); these are irregular mines usually >1 × 0.5 cm (i.e., not the narrow tunnels of "serpentine" mines). These are most often associated with miners of the genus *Nemorimyza* (Diptera: Agromyzidae), but similar blotch mines can be formed on plants in the Asteraceae family by the larvae of *Sumitrosis* (Coleoptera: Chrysomelidae), *Microrhopala* (Coleoptera: Chrysomelidae), and *Cremastobombycia* (Lepidoptera: Gracillariidae). We collected mines that seemed freshly vacated (the mine and the surrounding tissue had not yet dried), or were still occupied by a miner larva, and kept each in its own plastic snap-cap 60 dram vial.

In the laboratory, for mines that were already vacated ($N = 8$), we cut out the mined portion of the leaf with a sterilized scalpel, placed each excised mine into a 1.5 mL vial, and stored it at −80 °C until DNA extraction. For mines with larvae inside ($N = 88$), we held each leaf in a vial with a damp cotton ball, at room temperature under natural light, until the mine was vacated. All the mines were vacated successfully. For 38 mines, we cut out and froze the mined portion of the leaf immediately upon finding it vacated; for a further 50, we held the leaves for two additional days in the vials before cutting out the mined portion.

We extracted genomic DNA (gDNA) from each preserved mine using an acetylmethylammonium bromide (CTAB) protocol for animal tissue (Grosberg et al. 1996). Mined leaf tissue was ground in liquid nitrogen before CTAB extraction. We used 500 µL of CTAB mix and allowed 12–16 hours for tissue digestion. The concentration of gDNA extracted was measured using Qubit assays with a broad range kit (Thermo Fisher Scientific, Montana, USA). Aliquots of each gDNA sample were prepared for PCR work adjusting their concentrations to 2 ng/µL.

We amplified the standard 658 bp barcode region of cytochrome oxidase 1 using Folmer et al.'s (1994) polymerase chain reaction (PCR) primers (forward LCOI1490: 5′-GGTCAACAAATCAT AAAGATATTGG-3′; reverse HCOI2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′; Integrated

Fig. 1. Blotch leaf mine from *Doellingeria umbellata*.

DNA Technologies, Coralville, Iowa, USA), which provide near-universal amplification for many insect orders (Pons 2006). Amplification was performed in a 30 µL volume with 4 ng DNA, 1× ThermoPol reaction buffer (New England Biolabs (NEB), Whitby, Ontario, Canada), 0.2 mM dNTPs (NEB), 1× purified BSA (stock is 100×; NEB, cat. no. B9001S) and 2 mM $MgSO_4$ (stock is 100 mM; NEB, cat. no. B1003S), 0.5 µmol/L forward and reverse primers, and 1.0 U *Taq* DNA polymerase (NEB). Thermal cycling conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 19 cycles at 95 °C for 30 s;

from 45 to 64 °C with an increment of 1 °C per cycle for 30 s; 72 °C for 1 min, followed by 29 cycles at 95 °C for 30 s, 45 °C for 30 s, 72 °C for 1 min; and a final extension at 72 °C for 5 min.

We checked successful amplification (or amplicons of the partial COI gene) by running aliquots on 1.5% agarose gels at 100 V for 35 min and checking for bands in the target size (600–700 bp). Some samples showed only faint bands, so we used gel extraction to re-amplify these. We excised 600–700-bp bands with GeneCatcher gel excision pipettor tips (Gel Company, San Francisco, California, USA; cat. no. PKB6.5) and extracted DNA using QIAEX II gel-extraction kits (QIAGEN, Toronto, Ontario, Canada; cat. no. 20051) following the manufacturer's instructions. We sent 32 successfully amplified samples for sequencing to the McGill University and Genome Quebec Innovation Centre (Montreal, Québec, Canada).

We aligned, edited, and trimmed the sequences to a length of 658 bp using ClustalO (Sievers et al. 2011). We performed a nucleotide sequence similarity search using BLASTn (Altschul et al. 1990) and cross-referenced sequences in GenBank (Benson et al. 2011) using BLASTn query (and matched them in the Barcode of Life Data System (BOLD; Rathasingham and Hebert 2013) to appropriate Barcode Index Number (BIN) clusters for identification (Ratnasingham and Hebert 2013), searching all barcode records on BOLD). We tested whether there was a significant difference in the rates of molecular identification of leaf miners between mines vacated in the lab and those collected empty using a Fisher's exact test in R (R Core Team 2013).

Results

Fourteen samples showed clear bands upon initial amplification, indicating successful amplification. We obtained clear bands for an additional 18 samples following gel extraction and re-amplification. Of these 32 amplified samples, 30 were returned with high-quality sequence data (allowing 658 bp sequence alignment and thus GenBank identification). Nineteen of these matched (99%–100% match) to agromyzid leaf miners (Diptera: Agromyzidae): 11 to *Nemorimyza posticata* and eight to unidentified Agromyzidae sp. (**Table 1**). Seven sequences matched with the fungus *Jaminaea angkoriensis* (Basidiomycota: Microstromatales), two with whiteflies (Hemiptera: Aleyrodidae) and two with tydeid mites (Trombidiformes: Tydeidae) (**Table 1**). All arthropod specimens could be assigned to a BIN in the BOLD database: 11 sequences matched with *Nemorimyza posticata*, one with *Phytomyza* sp., seven with unidentified Agromyzidae spp., two with unidentified aleyrodid whiteflies, and two with mites in the genus *Tydeus*. The seven *Jaminaea angkoriensis* specimens could not be placed in a BIN because *Jaminaea* BOLD data because these specimens are a fungus and COI gene is not the barcode gene for fungi.

Amplification success did not depend on the timing of DNA extraction ($P = 0.15$). Two of the eight (25%) already-vacated mines, four of the 38 (11%) mines that were dissected immediately after being vacated, and 13 of the 50 (26%) mines vacated 2 days prior allowed successful identification of leaf-mining flies.

Discussion

Our extraction led to the successful amplification and sequencing of 19 leaf miners from our 96 empty mines (19%). Our mines were relatively fresh (40% were vacated for a day, 52% were vacated for just 2 days, with the remainder vacated for intervals that are unknown but likely measured in days at most). While we found no significant difference in amplification success among these categories, Derocles et al. (2015) noted that amplification success declined with time a leaf mine had been empty. We suspect that our amplification success would, similarly, have been lower if we had collected older mines. Time since the DNA was deposited seems to be a major determinant of amplification success in other systems as well (e.g., for mammalian scat, 3 days between deposition and DNA extraction made DNA much more difficult to identify; Santini et al. 2007). Storage conditions between collection

and extraction can also influence amplification success, as DNA degradation can be minimized by protection from light, preservation in regularly changed 90% ethanol (vs. 70%), and the storage of ethanol-preserved material in a freezer (Zimmermann et al. 2008).

Table 1. Leaf mine collection states (V = mine vacated in the field, I = mine dissected immediately in the lab, or L = mine dissected 2 days after being vacated in the lab), GenBank accession numbers, and identification results in GenBank and BOLD databases for DNA amplified from empty leaf mines with associated BOLD BIN cluster for each sample.

			GenBank		BOLD		
Sample ID	State	Accession #	ID	Similarity (%)	ID	Similarity (%)	BIN
EM_07	V	KX810685	Aleyrodidae sp.	99.7	Aleyrodidae sp.	99.6	BOLD:AAZ8499
EM_21	**V**	**KX810703**	***Nemorimyza posticata***	**100**	***N. posticata***	**99.9**	**BOLD:AAG9234**
EM_23	**V**	**KX810676**	**Agromyzidae sp.**	**100**	**Agromyzidae sp.**	**100**	**BOLD:AAG4743**
EM_32	L	KX810692	*Jaminaea angkoriensis*	85.1	*J. angkoriensis*	81.6	NA
EM_37	**L**	**KX810681**	**Agromyzidae sp.**	**100**	**Agromyzidae sp.**	**100**	**BOLD:AAG4743**
EM_39	**L**	**KX810680**	**Agromyzidae sp.**	**99.8**	**Agromyzidae sp.**	**99.8**	**BOLD:AAG4743**
EM_44	**L**	**KX810702**	***Nemorimyza posticata***	**99.8**	***N. posticata***	**99.5**	**BOLD:AAG9234**
EM_46	**L**	**KX810679**	**Agromyzidae sp.**	**99.8**	**Agromyzidae sp.**	**99.8**	**BOLD:AAG4743**
EM_49	**I**	KX810678	**Agromyzidae sp.**	**100**	***Phytomyza* sp.**	**100**	**BOLD:AAG4775**
EM_50	**L**	**KX810701**	***Nemorimyza posticata***	**99.8**	***N. posticata***	**99.5**	**BOLD:AAG9234**
EM_51	L	KX810684	Aleyrodidae sp.	99.5	Aleyrodidae sp.	99.6	BOLD:AAZ8499
EM_58	**L**	**KX810700**	***Nemorimyza posticata***	**99.5**	***N. posticata***	**99.4**	**BOLD:ACJ0616**
EM_61	L	KX810705	Tydeidae sp.	99.4	*Tydeus* sp.	97.6	BOLD:AAH0991
EM_67	I	KX810704	Tydeidae sp.	99.4	*Tydeus* sp.	97.6	BOLD:AAH0991
EM_69	**I**	**KX810693**	***Nemorimyza posticata***	**99.7**	***N. posticata***	**99.4**	**BOLD:AAG9234**
EM_72	**L**	**KX810698**	***Nemorimyza posticata***	**99.7**	***N. posticata***	**99.5**	**BOLD:AAG9234**
EM_75	L	KX810691	*Jaminaea angkoriensis*	91.8	*J. angkoriensis*	84.6	NA
EM_78	**L**	**KX810682**	**Agromyzidae sp.**	**100**	**Agromyzidae sp.**	**100**	**BOLD:AAG4743**
EM_86	**L**	**KX810697**	***Nemorimyza posticata***	**99.8**	***N. posticata***	**99.5**	**BOLD:AAG9234**
EM_88	I	KX810687	*Jaminaea angkoriensis*	91.8	*J. angkoriensis*	84.7	NA
EM_89	I	KX810690	*Jaminaea angkoriensis*	85.1	*J. angkoriensis*	81.7	NA
EM_92	**L**	**KX810677**	**Agromyzidae sp.**	**100**	**Agromyzidae sp.**	**100**	**BOLD:AAG4743**
EM_95	L	KX810686	*Jaminaea angkoriensis*	91.8	*J.angkoriensis*	84.7	NA
EM_96	L	KX810689	*Jaminaea angkoriensis*	85.1	*J. angkoriensis*	82.0	NA
EM_98	**L**	**KX810683**	**Agromyzidae sp.**	**99.8**	**Agromyzidae sp.**	**99.8**	**BOLD:AAG4743**
EM_102	**I**	**KX810696**	***Nemorimyza posticata***	**99.7**	***N. posticata***	**99.4**	**BOLD:AAG9234**
EM_105	**L**	**KX810695**	***Nemorimyza posticata***	**99.8**	***N. posticata***	**99.5**	**BOLD:AAG9234**
EM_106	L	KX810688	*Jaminaea angkoriensis*	91.8	*J. angkoriensis*	84.6	NA
EM_116	**L**	**KX810694**	***Nemorimyza posticata***	**99.8**	***N. posticata***	**99.5**	**BOLD:AAG9234**
EM_U	**I**	**KX810699**	***Nemorimyza posticata***	**99.3**	***N. posticata***	**99.4**	**BOLD:AAG9234**

Note: Leaf-mining taxa are in bold.

Of the 19 samples we identified as leaf miners, seven could not be assigned to a specific species because the leaf miner barcode library is incomplete. This is neither surprising nor worrisome, as barcode libraries continue to be developed and new leaf miner taxa continue to be discovered (J. Mlynarek, personal observation, 2015). When a method like ours yields a sequence with no barcode match, it has identified an unassigned molecular taxonomic unit, and the population from which those mines were sampled are identified as rewarding for further collection.

We also identified non-leaf-mining taxa from 11 samples: a fungus, one or more whiteflies, and one or more mites. We do not know whether these taxa were associated with the empty mines or just with the leaves. Making this distinction would require additional amplifications from un-mined leaf tissue, which we did not undertake, because our objective was to recover and identify leaf miner DNA. Non-target amplifications are not surprising, given our use of a universal insect primer. Workers wishing to avoid such amplifications can, of course, use more specific primers. However, non-target amplifications can also be seen as a source of additional data. Environmental DNA methods are increasingly being used to assess communities associated with soils, mycorrhizae, or leaves (Taberlet et al. 2012), including bacterial communities of leaves (Redford and Fierer 2009) and fungal communities of leaves (Kembel and Mueller 2014). With appropriate controls and additional primers, our extraction method could likely be applied to compare arthropod and microbial communities associated with herbivore-attacked and unattacked leaves and to examine succession in such communities post-herbivory. It is largely unknown whether non-insect associates of herbivore-damaged leaves contribute to the impact of herbivory (or perhaps mitigate it), but DNA-based identification methods may help make such inferences possible.

Our identification success rate of 19% may seem modest; indeed, for applications requiring identifications from every mine, collection of mines with larvae still present remains a necessity. However, in many applications (such as monitoring for invasive miners, identification of new pests, deducing leaf miner host ranges, or the detection of cryptic species), all that is required is the detection of the identified leaf miners from some mines. In such cases, our method can provide excellent power because plants under leaf miner attack often have multiple mines per plant (or even per leaf). For example, Godfray (1985) observed on average 24.5 leaf miners per 1000 leaves on a sample of 32 herbaceous plant species, with *Rumex crispus* having as many as 253 miners per 1000 leaves. Collecting multiple mines at a site, and even from a single plant, should then be straightforward. With a 19% success rate per mine, very high overall detection success rates can be attained: 65% given just five mines, 88% given 10 mines, and 99% given 20 mines (probability of at least one successful identification for n mines $= 1 - 0.81^n$). Of course, further methodological development is likely to increase our success rate, further improving power of detection.

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Author contributions

Conceived and designed the study: JJM, SBH. Performed the experiments/collected the data: JJM, J-HK. Analyzed and interpreted the data: JJM, J-HK. Contributed resources: JJM, SBH. Drafted or revised the manuscript: JJM, J-HK, SBH.

Competing interests

SBH is currently serving as a Subject Editor for FACETS, but was not involved in review or editorial decisions regarding this manuscript.

Data accessibility statement

All relevant data are within the paper and available in GenBank. Barcodes are available from https://www.ncbi.nlm.nih.gov/genbank/; Accession # KX810676-KX810705.

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