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DNA Barcoding to Identify All Life Stages of Holocyclic Cereal Aphids (Hemiptera: Aphididae) on Wheat and Other Poaceae

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ABSTRACT The cytochrome *c* oxidase subunit I (COI) gene of the mitochondrial DNA was sequenced in eight holocyclic monoecious aphids (Hemiptera: Aphididae) that occur on wheat, *Triticum aestivum* L.; barley, *Hordeum vulgare* L.; oat, *Avena sativa* L.; and sorghum, *Sorghum bicolor* (L.) Moench in the United States. The first 640 bp of the 5' end were considered as a DNA barcoding technique for species identification. DNA barcoding successfully differentiated *Schizaphis graminum* (Rondani), *Diruaphis noxia* (Kurdjumov), *Diruaphis tritici* (Gillette), *Diruaphis frequens* (Walker), *Diruaphis mexicana* (McVicar Baker), *Sipha flava* (Forbes), *Sipha elegans* del Guercio, and *Sitobion avenae* (F.). In addition to the above-mentioned monoecious species, the common cereal aphids *Rhopalosiphum padi* (L.) and *Rhopalosiphum maidis* (Fitch) were included and successfully differentiated. DNA barcoding is a reliable alternative to traditional morphology in the identification of cereal aphids and their various life stages and morphs, including eggs. The application of DNA barcoding to aphid eggs found on grasses will be able to confirm whether *D. noxia* is now reproducing sexually in the United States after 20 yr of asexual reproduction.

KEY WORDS holocyclic reproduction, DNA barcoding, *Diruaphis*, *Schizaphis*, aphid

Aphids (Hemiptera: Aphididae) are among the principal pests of cereal grains in the United States. Cereal grains mainly refer to wheat, *Triticum aestivum* L.; barley, *Hordeum vulgare* L.; and oat, *Avena sativa* L. Sorghum, *Sorghum bicolor* (L.) Moench, also is sometimes considered. Besides causing direct damage, aphids also are vectors of plant viruses, especially barley yellow dwarf and cereal yellow dwarf. Cereal aphids may be divided into two groups based on their reproductive cycle: anholocyclic and holocyclic.

Anholocyclic species lack a sexual cycle and populations are comprised of viviparous females that give birth to live young via apomictic parthenogenesis (Dixon 1985). Holocyclic species reproduce both asexually and sexually. Several apomictic parthenogenetic generations are followed by the production of males and oviparous females that mate and lay eggs that overwinter (Dixon 1985). Within the holocyclic form of reproduction, there are species that are heteroecious, i.e., they host alternate between the sexual and asexual stages, and monoecious, in which they use the same host through out their life stages (Dixon 1985).

Cereal aphids with monoecious holocycly are of primary interest because genetic recombination during the sexual cycle may lead to new biotypes capable of injuring resistant crop cultivars (Puterka and Peters 1990). In the United States, the most important monoecious holocyclic species associated with cereals and grasses are greenbug, *Schizaphis graminum* (Rondani); Russian wheat aphid, *Diruaphis noxia* (Kurdjumov); *Diruaphis tritici* (Gillette); *Diruaphis frequens* (Walker); *Diruaphis mexicana* (McVicar Baker); yellow sugarcane aphid, *Sipha flava* (Forbes); *Sipha elegans* del Guercio; and English grain aphid, *Sitobion avenae* (F.).

Although *D. noxia* was reported as holocyclic in its native range, it was only thought to be anholocyclic in the United States (Kiriatic et al. 1990). Oviparae and unfertilized eggs were found in the United States, but not males (Kiriatic et al. 1990). Beginning in 2003, there was a rapid occurrence of *D. noxia* biotypes and expansion of their range (Haley et al. 2004, Burd et al. 2006, Puterka et al. 2007). There is strong evidence that no additional introductions of *D. noxia* occurred and the biotype arose from the extant population (Shufran et al. 2007, Shufran and Payton 2009). Therefore, genetic recombination during the holocycle was thought to have been a likely source of the biotypes (Puterka et al. 2007). Anholocyclic populations of invasive aphids have become holocyclic as they expanded their range, e.g., *Therioaphis trifolii* forma

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Table 1. Insect material used to sequence the DNA barcoding region of the COI gene and GenBank accession numbers

Species	Biotype, (COI haplotype)	Location	Date	Accession no.	Reference
<i>D. noxia</i>	I	Fort Collins, CO	May 2003	HQ392571	Shufran et al. (2007)
<i>D. tritici</i>	NA ^a	Meeker, CO	25 June 1996	HQ392589	Shufran et al. (2007)
<i>D. mexicana</i>	NA	Grand Junction, CO	June 1998	HQ392570	NA
<i>D. frequens</i>	NA	Grand Junction, CO	June 1998	HQ392569	NA
<i>S. graminum</i>	B	ARS Laboratory Culture	Feb. 1997	HQ392572	Shufran et al. (2000)
	C	ARS Laboratory Culture	Feb. 1997	HQ392573	Shufran et al. (2000)
	E	ARS Laboratory Culture	Feb. 1997	HQ392575	Shufran et al. (2000)
	F	ARS Laboratory Culture	Feb. 1997	HQ392576	Shufran et al. (2000)
	G	ARS Laboratory Culture	Feb. 1997	HQ392577	Shufran et al. (2000)
	H	ARS Laboratory Culture	Feb. 1997	HQ392578	Shufran et al. (2000)
	I	ARS Laboratory Culture	Feb. 1997	HQ392582	Shufran et al. (2000)
	J	Parma, ID	Unknown	HQ392583	Beregovy and Peters (1995)
	K	ARS Laboratory Culture	Feb. 1997	HQ392584	Shufran et al. (2000)
	NY	ARS Laboratory Culture	Feb. 1997	HQ392585	Shufran et al. (2000)
	<i>Paspalum vaginatum</i> Swartz	Belle Glade, FL	Nov. 2004	HQ392586	Nuessly et al. (2008)
	E, (I)	Okeene, OK	13 Mar. 2008	HQ392579	NA
	Unknown, (II)	Okeene, OK	13 Mar. 2008	HQ392580	NA
	B, (III)	Okeene, OK	13 Mar. 2008	HQ392581	NA
<i>S. elegans</i>	NA	Meade, CO	Aug. 1996	HQ392587	NA
<i>S. flava</i>	NA	Stillwater, OK	July 1994	HQ392588	NA
<i>S. avenae</i>	NA	ARS Laboratory Culture	April 2000	HQ392574	NA
<i>R. maidis</i> ^b	NA	Unknown	Unknown	EU701890	Footit et al. (2008)
<i>R. padi</i> ^b	NA	Ontario, Canada	Unknown	EU701894	Footit et al. (2008)

^a Not applicable.

^b Previously published sequence.

maculata (Buckton) (Manglitz et al. 1966). Although the occurrence of new biotypes was circumstantial evidence that *D. noxia* became holocyclic in the United States, males, fertilized eggs, or both were not found to support this hypothesis.

Identification of cereal aphids and *Diuraphis* spp. is traditionally based on morphological characters, and although several keys exist, none can separate all species and all life stages (Aalbersberg et al. 1987, Stoetzel 1987, Pike et al. 1991, Halbert et al. 1992, Blackman and Eastop 2000, Miller et al. 2005). Furthermore, aphid eggs cannot be identified without rearing in the laboratory and then keying out the fundatrices, subsequent viviparous offspring, or both. This process is time-consuming and laborious, and egg hatch is often unsuccessful. In addition, most fundatrices of holocyclic aphids are not described. Rearing eggs found in nature is not a reliable method for identification.

DNA barcoding (Hebert et al. 2003) is a technique that is applicable to solving the problem of cereal aphid identification, independent of life stage or undescribed morphs. The technique involves DNA sequencing approximately a 638-bp region of the 5' end of cytochrome *c* oxidase I (COI) gene in the mitochondrial (mt)DNA. This COI region has been successfully amplified and sequenced from eggs of *S. graminum*, *D. tritici*, *D. frequens*, and *D. mexicana* (K.A.S., unpublished data). Footit et al. (2008) successfully used DNA barcoding to discriminate >300 species of aphids. We describe the use of DNA barcoding to differentiate holocyclic cereal aphids as a reliable alternative to traditional morphology.

Materials and Methods

The entire COI gene was polymerase chain reaction (PCR) amplified and DNA sequenced in the taxa described in Table 1. The following primers were used to amplify aphid DNA: LepF and LepR (Hajibabei et al. 2006); C1-J-1718, C1-N-2191 "Nancy", C1-N-2329 "K525", and L2-N-3014 "Pat" (Simon et al. 1994); and two primers we designed "Bert" 5'-TTAATAATA-ATAATTTGTAGA-3' and "Ernie" 5'-ATAATCTG-TATATCGTCGTG-3'.

DNA was isolated from single aphids using the *prep-GEM* extraction kit (ZyGEM Corp. Ltd., Hamilton, New Zealand). We added 100 ng of template DNA to 25- μ l polymerase chain reactions (PCRs) with the following conditions: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM dNTPs, 2.5 mM MgCl₂, 20 pmol of each primer, and 1.5 U of GoTaq DNA polymerase (Promega, Madison, WI). A PTC-100 thermal controller (MJ Research, Watertown, MA) was used with the following program steps: 1) 96°C 3 min; 2) 94°C 30 s; 3) 50°C 30 s; 4) 72°C 1 min; 5) cycle to step 2, 34 times; 6) 72°C 5 min; and 7) 4°C hold. The presence of PCR amplicons of correct size was determined by standard agarose gel electrophoresis as described by Sambrook et al. (1989).

PCR products were direct purified using the Wizard SV Gel and PCR Clean-Up System (Promega). DNA sequences for both strands were obtained by using BigDye (Applied Biosystems, Foster City, CA) terminated reactions with an ABI 3700 DNA analyzer at the Recombinant DNA/Protein Resource Facility, Oklahoma State University, Stillwater, OK. The COI fragments from each specimen were subjected to 3 \times coverage and assembled with Seq Man Pro in the

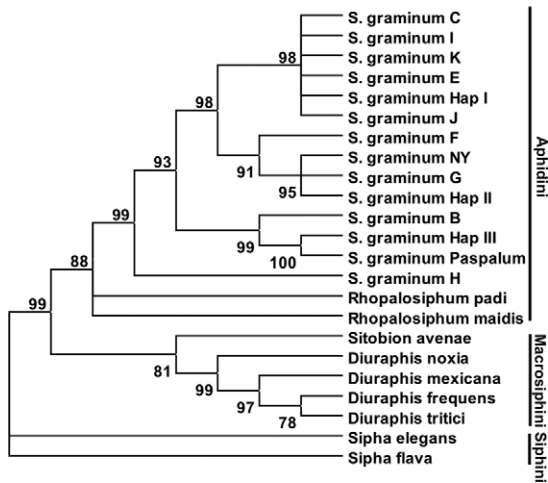


Fig. 1. Nonrooted, neighbor-joining tree of holocyclic cereal aphids, with *R. padi* and *R. maidis* included. Produced using 500 bootstrap replications with a significance value of 50% supported branches.

Lasergene version 8.0 (DNASTAR, Madison, WI) package. DNA sequences were aligned by the ClustalW method (Thompson et al. 1994) with Megalign in the Lasergene version 8.0 (DNASTAR). COI sequences from two common cereal aphids not holocyclic on grasses, *Rhopalosiphum padi* (L.) and *Rhopalosiphum maidis* (Fitch) (Footitt et al. 2008), were included (Table 1). A nonrooted, neighbor-joining dendrogram was produced with 500 bootstrap replications and a branch significance value of 50% by using MEGA version 4 (Tamura et al. 2007). DNA sequences were submitted to GenBank, and accession numbers are listed in Table 1.

Results and Discussion

The entire (≈1.4-kb) COI was sequenced from holocyclic cereal aphids in the United States. An alignment of only the accepted standard region for DNA barcoding (Hebert et al. 2003), i.e., 640 bp of the 5' end was produced (data not shown). We included sequences from two common species found on wheat and barely, *R. padi* and *R. maidis*. Each species and *S. graminum* mtDNA haplotypes could be distinguished by DNA sequence, demonstrating the power and utility of DNA barcoding as an identification technique (Fig. 1). Neighbor-joining correctly grouped species into their respective subfamily and tribe, with 99% bootstrap support (Fig. 1). Similar results were found by Footitt et al. (2008) with 300 aphid species.

Intraspecific DNA sequence variation varied between species. Footitt et al. (2008) reported an average of 0.2% divergence within species. We found a mean of 2.54%, with range of 0.0–5.7% divergence within *S. graminum* when including all three COI haplotypes (Shufran et al. 2000) and biotypes (Table 2). Despite the large variation in sequence divergence within *S. graminum*, the species was grouped to its own clade with 99% bootstrap support (Fig. 1).

Table 2. Sequence variation within *S. graminum* 640-bp COI DNA barcoding region

Group	n	Mean % divergence	Range % divergence
All biotypes and COI haplotypes	14	2.54	0.0–5.7
All biotypes and COI haplotypes except H	13	2.15	0.0–4.7
COI haplotype I	6	0.37	0.0–0.9
COI haplotype II	4	0.55	0.0–1.1
COI haplotype III	3	0.73	0.0–1.1

When the rare *S. graminum* biotype H was omitted from the data set, there was a still a mean of 2.15% and a range of 0.0–4.7% in sequence divergence (Table 2). The variation was reduced considerably when considering sequences within mtDNA haplotypes (Table 2). Footitt et al. (2008) only considered a single *S. graminum* specimen. Our results demonstrate the need for extensive investigation of intraspecific variation to verify DNA barcoding results. Alternatively, we found the complete opposite with *D. noxia*, i.e., there was none to very little intraspecific variation in *D. noxia* from North America.

Shufran and Payton (2009) sequenced 27 *D. noxia* of different geographic origin (within the United States) and biotype. Their sequences overlapped ≈400 bp of the 3' DNA barcoding region used in this study, and zero variation was found (data not shown). Similarly, Shufran et al. (2007) found zero DNA sequence variation in the COI among >50 individuals of *D. noxia* also from the United States. Therefore, we did not DNA sequence the terminal 5' 240-bp end of multiple *D. noxia* specimens. Because zero variation was found in DNA sequences of the COI, identification of *D. noxia* via DNA barcoding would be very reliable. Any aphid eggs found on wheat or barley in the United States could be reliably identified using this method. DNA barcoding has the potential to definitively answer the question of whether *D. noxia* is holocyclic and overwintering as eggs in the United States. Because the COI is haploid and maternally inherited, its DNA sequence is independent of life stage or morph. Previously, we extracted DNA from the eggs of *S. graminum*, *D. tritici*, *D. mexicana*, and *D. frequens*; PCR amplified the COI; and sequenced it (unpublished data). The sequences obtained from eggs were identical to those obtained from apterous and alate viviparous morphs.

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