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Barcode haplotype variation in north American agroecosystem lady beetles (Coleoptera: Coccinellidae)

MATTHEW H. GREENSTONE,* NATALIA J. VANDENBERG† and JING H. HU*
*United States Department of Agriculture, Agricultural Research Service, Invasive Insect Biocontrol and Behavior Laboratory, Beltsville, MD 20705, USA, †United States Department of Agriculture, Agricultural Research Service, Systematic Entomology Laboratory, Beltsville, MD 20705, USA

Abstract

DNA barcodes have proven invaluable in identifying and distinguishing insect pests, most notably for determining the provenance of exotic invasives, but relatively few insect natural enemies have been barcoded. We used Folmer *et al.*'s (1994) universal invertebrate primers and Hebert *et al.*'s (2004) for Lepidoptera, to amplify 658 bp at the 5′ end of the mitochondrial cytochrome oxidase c subunit I (COI) gene in five species of lady beetles from crop fields in six states in the US Mid-Atlantic, Plains and Midwest: three native species, *Hippodamia convergens* Guérin-Méneville, *H. parenthesis* (Say) and *Coleomegilla maculata* (De Geer); and two exotic species, *Harmonia axyridis* (Pallas) and *Coccinella septempunctata* Linnaeus. Sequence divergences within species were low, never exceeding 0.9% (Kimura 2-parameter distances). Sequence divergences between the two *Hippodamia* species ranged from 14.7 to 16.4%, mirroring the relationships found for other arthropod taxa. Among the exotic species, *C. septempunctata* sequences were as variable as those of the three native species, while *H. axyridis* populations comprised a single haplotype. Limited data on two *Coleomegilla* subspecies, *C. m. lengi* Timberlake and *C. m. fuscilabris* (Mulsant), are consistent with their belonging to the same species, although morphological and reproductive data indicate that they represent separate species. Our results support the general utility of COI barcodes for distinguishing and diagnosing coccinellid species, but point to possible limitations in the use of barcodes to resolve species assignments in recently divergent sibling species.

Keywords: *Coccinella*, Coccinellidae, *Coleomegilla*, cytochrome oxidase I, DNA barcode, *Harmonia*, *Hippodamia*

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Introduction

DNA barcodes have proven invaluable in identifying and distinguishing insect pests, including determining the provenance of exotic invasives (Gleeson *et al.* 2000; Ball & Armstrong 2006; Havill *et al.* 2006; Scheffer *et al.* 2006), but relatively few insect natural enemies have been barcoded. Lady beetles (Coleoptera: Coccinellidae) are important insect natural enemies in agroecosystems worldwide (Hodek & Honěk (1996). Our goal in this study was to develop the first barcodes for common North American coccinellids and evaluate their utility for diagnostic purposes.

We selected five species of lady beetles from wheat, sorgum, alfalfa, soybean and cotton fields in six states in the US Plains, Midwest and Mid-Atlantic: three native species, *Hippodamia convergens* Guérin-Méneville, *H. parenthesis* (Say) and *Coleomegilla maculata* (De Geer); and two exotic species, *Harmonia axyridis* (Pallas) and *Coccinella septempunctata* Linnaeus. Additionally, we compared populations of two *C. maculata* subspecies: *C. m. lengi* Timberlake and *C. m. fuscilabris* (Mulsant) (*sensu* Gordon 1985). These species were selected for study because they are widespread and common in the US, important biological control agents and, for various reasons, represent interesting subjects for both historical and prospective genetic studies. All belong to the tribe Coccinellini, which contains the majority of the larger, showier lady beetles.

Hippodamia spp. were popular subjects among early geneticists because of phenotypic variations in their elytral colour patterns. In contrast, most modern geneticists have favoured more easily reared subjects with shorter generation times, e.g., *Drosophila* spp., for insect variability studies. As a result, our understanding of the genetics of native North American lady beetles has progressed relatively little. A study by Timberlake (1919) showed that *H. parenthesis* could produce F1 hybrids

Correspondence: Matthew H. Greenstone, Fax: (301) 504 5104;
E-mail: matt.greenstone@ars.usda.gov

with *H. lunatomaculata* Motschulsky under laboratory conditions and that the offspring exhibited intermediate genitalic characteristics. Chapin (1946) studied colour pattern variation and genitalic characteristics across the North American representatives of *Hippodamia*, and Shull (1943, 1945, 1946a,b, 1948) made a number of experimental crosses among the more closely related congeners. For example, *H. convergens* was shown to hybridize with another native species, *H. quinquesignata* (Kirby), and even produced an F2 generation and viable offspring in successive back crosses to both parent species. Modern studies have focused on the behaviours and distributions of the aforementioned species, but data on the genetic variability of field collected populations, and modern genetic studies on native North American lady beetles as a whole, are largely lacking. Unfortunately, the feasibility of such studies is declining rapidly with the introduction of exotic invasive lady beetles and the subsequent decline of many North American natives (reviewed in Gardiner *et al.* 2009).

Our third native species, the spotted pink lady beetle, also known as the pink-spotted lady beetle, spotted lady beetle or pink lady beetle, has long been considered a single species, *Coleomegilla maculata*, with a number of subspecies and varieties widely distributed throughout the Neotropics and much of the US and southern Canada. Unlike *Hippodamia*, *Coleomegilla* is characterized by conservative variations in both elytral colour patterns and male genitalia, making species determinations difficult for the layperson. Gordon (1985) recognized three subspecies in North America, but more recent allozyme analyses and breeding experiments (Coll *et al.* 1994; Krafsur *et al.* 1995; Krafsur & Obrycki 2000; Pérez & Hoy 2002) indicate that *C. maculata* is a species complex with two species in America north of Mexico and at least one additional species south of the Mexican border. Like the aforementioned authors, we continue to use the name *Coleomegilla maculata* and the subspecies designations of Gordon (1985) for North American members of this genus pending a formal revision, including comparison with the nominate subspecies *C. m. maculata* from South America.

Both the Palearctic *C. septempunctata* and Asian *H. axyridis* were intentionally released as biological control agents many times in the US, but the exact origin of established populations remains a matter of debate. Progeny of *C. septempunctata* collected in India, France, Italy, Norway and Sweden were repeatedly released in the eastern and western US as early as 1957, and in eastern Canada from 1959 to 1960 (Gordon 1985; Wheeler & Hoebeke 1995). Established populations of *C. septempunctata* were first detected in New Jersey and Quebec in 1973 and attributed to intentional releases against aphid pests (Schaefer *et al.* 1987), adventive establishment associated with air travel (Angalet & Jacques 1975) or transoceanic commerce (Schaefer *et al.* 1987; Schaefer & Dysart 1988; Day *et al.* 1994; Wheeler & Hoebeke 1995).

Populations of *H. axyridis* collected in Japan, Russia, Korea and the former Soviet Union were imported for propagation and repeatedly released in multiple states within the US beginning in 1916. Despite repeated sampling, established populations were not detected until 1988 in Louisiana (Chapin & Brou 1991), 1990 in Mississippi, 1991 in Georgia, 1992 in Arkansas, 1993 in Oregon, and 1994 in Iowa and Illinois (Tedders & Schaefer 1994; Krafsur *et al.* 1997). Tedders & Schaefer (1994) suggest that two independent populations were established, one in Louisiana and another in Georgia, as the result of releases made in 1979 and 1980. Day *et al.* (1994) attribute the establishment to accidental introductions occurring at seaports and point out that the established populations were found hundreds of kilometres from release sites and after an interval of 8–10 years.

Both *C. septempunctata* and *H. axyridis* have become widespread throughout most of the US, and their presence has been associated with the decline in abundance of many native North American lady beetle species (Wheeler & Hoebeke 1995; Evans 2000; Turnock *et al.* 2003; Hesler & Kieckhefer 2008). Krafsur *et al.* (1992, 1997) studied the genetic diversity of the North American populations of both of these exotics in an attempt to answer questions about their origins and routes of entry into the US, but they were unable to distinguish between intentional and adventive introductions based on available data. On the other hand, the genetic similarities observed among the widely distributed North American *H. axyridis* populations (Krafsur *et al.* 1997) support the hypothesis of a single introduction followed by rapid spread.

Materials and methods

Insects

The animals were collected by hand or sweep netting, placed immediately into 80% EtOH, sent to the senior author and maintained at −20 °C until ready for DNA extraction. Collecting localities are given in Table 1. At one of the Kentucky sites, Silver Lake Farm, the collection was from outside of the crop, in *Conium maculatum* L. (poison hemlock). Also, all specimens of *C. m. fuscilabris* were collected on *Nuphar lutea* (L.) (yellow pond lily).

The identities of all animals were verified by the second author prior to molecular analysis.

Molecular methods

DNA extraction and preliminary amplification. DNA was extracted from whole animals by the method of

Table 1 Collecting localities and barcode and vouchering data for coccinellids in this study

Locality	State, county	Crop	Species	N	K-2p	GenBank no.	USNM carcass no.	USNM DNA no.
Altus	OK, Jackson	Cotton	*Hippodamia convergens*	13	0.0–0.9	JF296080– 2F296092	00761000–00761012	00530943–00530955
Borger	TX, Hutchinson	Wheat	*H. convergens*	6	0.0–0.3	JF296093–JF296098	00761013–00761018	00530956–00530961
Briggsdale	CO, Weld	Wheat	*H. convergens*	14	0.0–0.3	JF296147–JF296160	00761019–00761032	00530962–00530975
			H. parenthesis	26	0.0–0.3	JF296238–JF296263	00761033–00761058	00530976–00531001
			Coccinella septempunctata	4	0.0	JF296221–JF296224	00761059–00761062	00531002–00531005
Brookings	SD, Brookings	Soybean	*Harmonia axyridis*	23	0.0	JF295977–JF295999	00761063–00761085	00531006–00531028
		Alfalfa	*C. septempunctata*	23	0.2–0.6	JF296198–JF296220	00761086–00761108	00531029–00531051
Burlington	OK, Alfalfa	Sorghum	*H. convergens*	3	0.0–0.3	JF296052–JF296054	00761109–00761111	00531052–00531054
Chickasha	OK, Grady	Sorghum	*C. maculata lengi*	5	0.2–0.6	JF296225–JF296229	00761112–00761116	00531055–00531059
			H. convergens	3	0.0–0.2	JF296049–JF296051	00761117–00761119	00531060–00531062
Hereford	TX, Deaf Smith	Wheat	*H. convergens*	25	0.0–0.8	JF296055–JF296079	00761120–00761144	00531063–00531087
Hughes Hollow	MD, Montgomery	*Nuphar*	*C. maculata fuscilabris*	3	0.0	JF2966230–JF296232	00761145–00761147	00531088–00531090
Lamar	CO, Prowers	Wheat	*H. convergens*	37	0.0–0.9	JF296012–JF296048	00761148–00761184	00531091–00531127
			H. parenthesis	25	0.0–0.2	JF296284–JF296308	00761185–00761209	00531128–00531152
			C. septempunctata	37	0.0–0.5	JF296161–JF296197	00761210–00761246	00531153–00531189
Lexington	KY, Fayette	Alfalfa	*C. maculata lengi*	5	0.0	JF296233–JF296237	00761247–00761251	00531190–00531194
			H. axyridis	9	0.0	JF296003–JF296011	00761252–00761260	00531195–00531203
Perryton	TX, Ochiltree	Wheat	*H. convergens*	18	0.0–0.6	JF296099–JF296116	00761261–00761278	00531204–00531221
Silver Lake Farm	KY, Harrison	*Conium*	*H. axyridis*	3	0.0	JF296000–JF296002	00761279–00761281	00531222–00531224
Springfield	CO, Baca	Wheat	*H. convergens*	30	0.0–0.3	JF296117–JF296146	00761282–00761311	00531225–00531254
			H. parenthesis	20	0.0–0.8	JF296264–JF296283	00761312–00761331	00531255–00531274
Tulia	TX, Swisher	Wheat		5				

K-2p, Kimura 2-parameter distance; USNM, US National Museum, Smithsonian Institution.

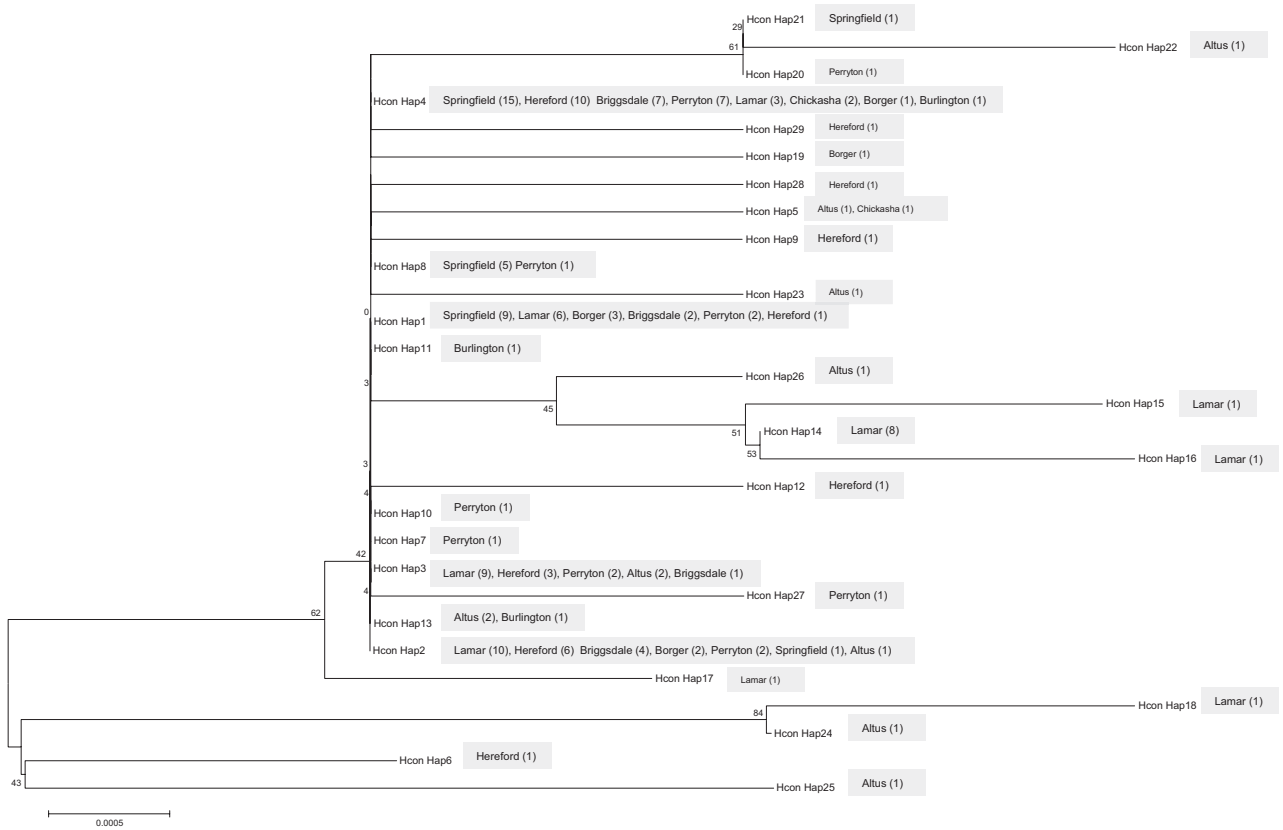

Fig. 1 Neighbour-joining phylogeny of *Hippodamia convergens* COI haplotype sequences. Complete locality data are presented in Table 1. Bootstrap support based on 1000 replications. Shaded boxes are collecting localities; numbers in parentheses are numbers of individuals per locality with each haplotype.

Rowley *et al.* (2007), which results in intact complete specimens suitable for vouchering, and amplified with two pairs of COI primers: universal invertebrate primers LCO1490 and HCO2198 (Folmer *et al.* 1994) and Lepidoptera primers LepF1 and LepR1 (Hebert *et al.* 2004).

Preliminary PCRs (50 μL) contained 2. 5× GoTaq buffer (Promega, Madison, WI, USA), 1.0 μM dNTPs, 1.0 μM of each primer, 5 μM $MgCl_2$ and 20 ng of genomic DNA. Thermocycling conditions for primers LepF1 and LepR1 followed Hebert *et al.* (2004). For amplification with primers LCO1490 and HCO2198, initial denaturation was for 3 min at 95 °C, followed by 44 cycles of 1 min at 95 °C, 2 min at 50 °C and 2 min at 72 °C; 5 min at 72 °C completed the programme.

The success of the reactions was checked by electrophoresis of 10 μL of the PCR/stop reaction in 1.5% agarose in 1.0× TAE buffer.

DNA sequencing. PCR products were purified using the Wizard PCR preps DNA purification system (Promega). PCRs for sequencing (BigDye Therminator v3.1; Applied Biosystems, Indianapolis, IN, USA) totalled 5.5 μL containing 0.5 μL BigDye reagent, 1.5 μL 5× sequencing buffer, 0.3 μL primer (20 μM stock), 1.2 μL H_2O and 2 μL purified DNA template. The thermocycling profile was initial denaturation for 1 min at 96 °C, followed by 44 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C; an indefinite hold at 10 °C completed the programme. PCR products were sequenced in two directions on an ABI3100 automated sequencer (Applied Biosystems). Editing and alignments were performed with Lasergene (DNAStar), utilizing the Clustal W algorithm for alignment. Nucleotide divergences were expressed as pair-wise per cent (Kimura 2-P distances), and phylogenies were generated by neighbour joining (Saitou & Nei 1987) with Mega4.1 (Tamura *et al.* 2007). Only individuals yielding bi-directional reads were used in the analysis.

Deposition of morphological and DNA vouchers, and DNA sequences. Extracted coccinellid carcasses were deposited as morphological vouchers, prepared according to standard museum practices, in the Insect and Mite Collection of the Smithsonian Institution, National Museum of Natural History (USNM) in Washington, DC; the

companion DNA samples were deposited in the USNM Tissue Collection. Voucher numbers for both carcasses and DNA are provided in Table 1. Haplotype sequences were deposited in GenBank; their accession numbers are also given in Table 1.

Results

Phylogenies for all species except *H. axyridis* are given in Figs 1–5. Within-species among-population sequence divergences were 0.0 for *C. m. fuscilabris* (the Hughes Hollow *C. maculata* population) and *H. axyridis*, 0.0–0.5% for *C. m. lengi* (all other *C. maculata* populations), 0.0–0.6% for *C. septempunctata* and *H. parenthesis*, and 0.0–0.9% for *H. convergens*. Sequence divergences between *H. parenthesis* and *H. convergens* sequences ranged from 14.7–16.4%, reflecting complete separation of the two species with 99% bootstrap support (Fig. 3), while divergences between the two *C. maculata* subspecies ranged from 0.0 to 0.8% and do not support separate species status (Fig. 4). Sequences of all of the *H. convergens* collected in a Tulia, Texas, wheat field diverged from conspecifics in other localities by more than 14%. When subjected to BLAST, the Tulia consensus was a 99.5% match to *Diuraphis noxia*, the Russian wheat aphid; these animals were therefore excluded from the analysis. A similarly divergent *H. parenthesis* sequence from a Briggsdale, Colorado wheatfield was 85.2% identical to *H. axyridis* and was therefore excluded from further analysis.

Discussion

A basic criterion for species-specific diagnostic utility of DNA barcodes is that intraspecific sequence variability always be less than that among species in the genus, with the most stringent test involving sister species (Moritz & Cicero 2004). Although the two *Hippodamia* species in this study are not sisters (Chapin 1946), the among-sequence distances within each of these species were invariably more than an order of magnitude less than those among them. This mirrors the situation for COI barcodes in other species of insects (Ball *et al.* 2005; Ball & Armstrong 2006; Rojo *et al.* 2006; Smith *et al.* 2006), Collembola (Hogg & Hebert 2004), spiders (Barrett & Hebert 2005) and Crustacea (Barber & Boyce 2006).

On the other hand, our limited sampling of North American populations of *Coleomegilla maculata* failed to recover the expected (Pérez & Hoy 2002) sister-group relationship between our Hughes Hollow, Maryland population of *C. m. fuscilabris* and samples of *C. maculata* from Lexington, Kentucky and Chickasha, Oklahoma (Fig. 4). Using Gordon's (1985) revision, members from

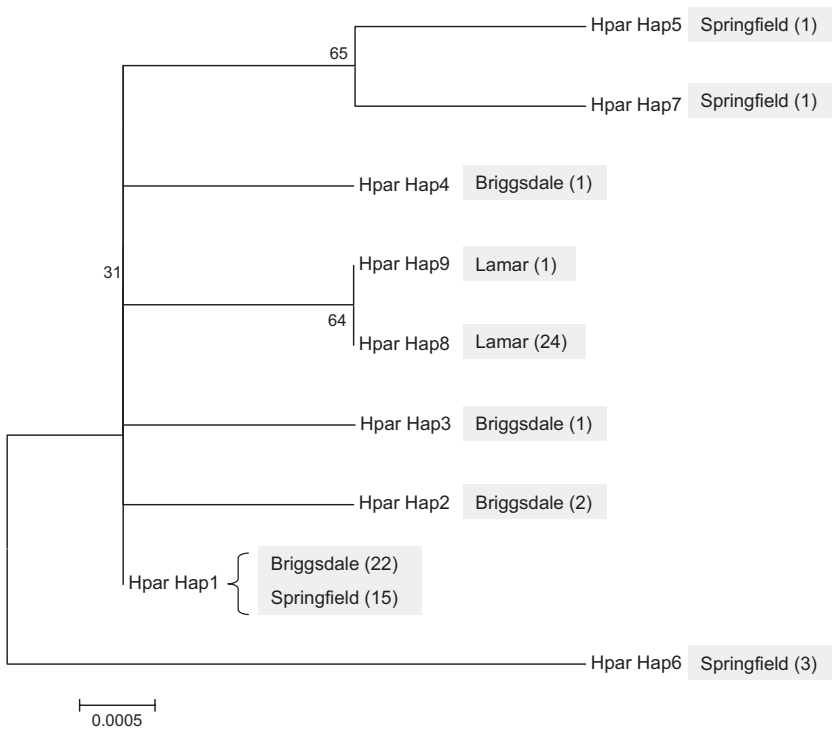

Fig. 2 Neighbour-joining phylogeny of *Hippodamia parenthesis* COI haplotype sequences. Complete locality data are presented in Table 1. Bootstrap support based on 1000 replications. Locality labels as in Fig. 1.

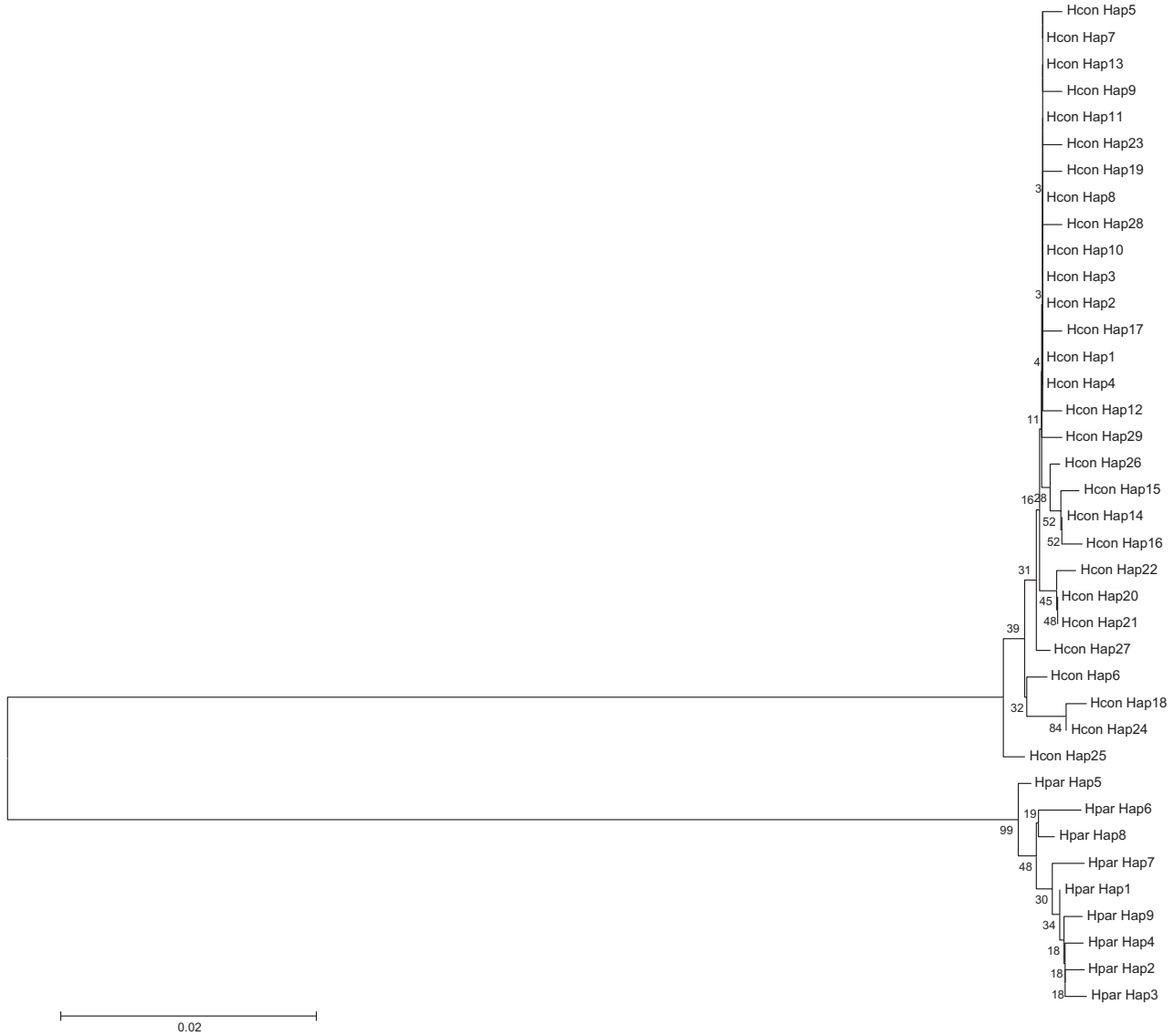

Fig. 3 Neighbour-joining phylogeny of all *Hippodamia* COI haplotype sequences. Complete locality data are presented in Table 1. Bootstrap support based on 1000 replications. Locality labels omitted for clarity.

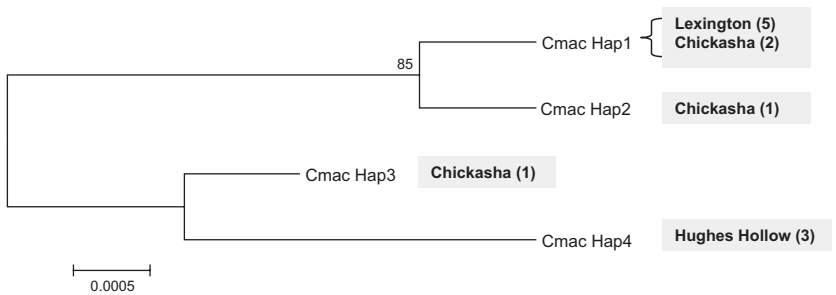

Fig. 4 Neighbour-joining phylogeny of *Coleomegilla maculata* COI haplotype sequences. Complete locality data are presented in Table 1. Bootstrap support based on 1000 replications. Locality labels as in Fig. 1. Hughes Hollow specimens are *C. maculata fuscilabris;* all other specimens are *C. m. lengi.*

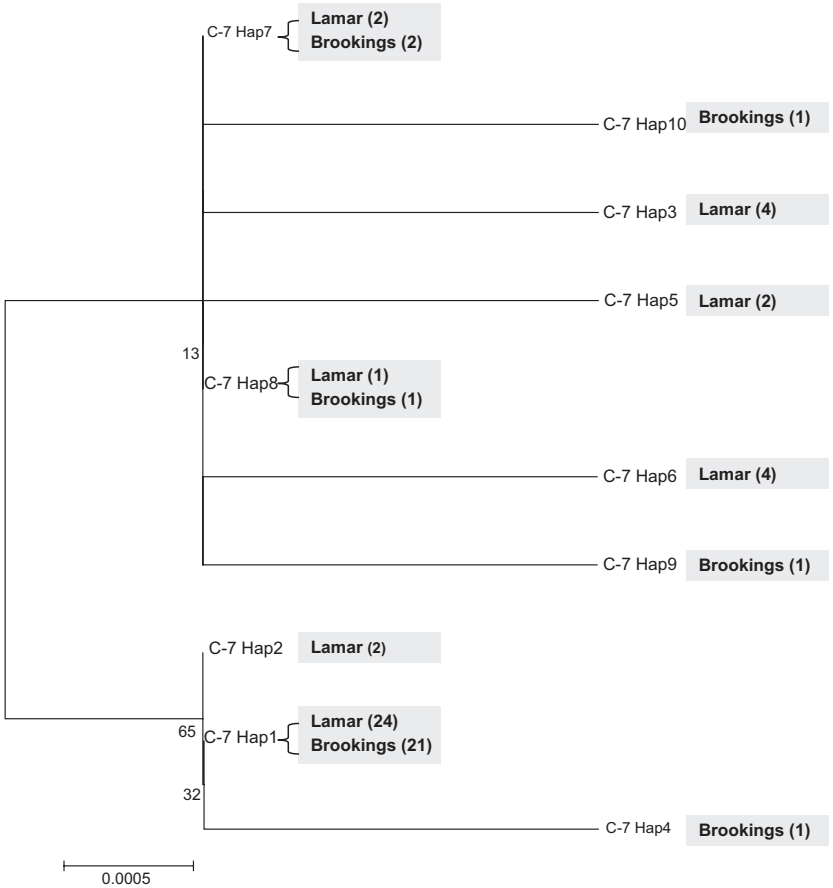

Fig. 5 Neighbour-joining phylogeny of *Coccinella septempunctata* COI haplotype sequences. Complete locality data are presented in Table 1. Bootstrap support based on 1000 replications. Locality labels as in Fig. 1.

both of the latter localities would be assigned to *C. m. lengi*, although the Chickasha sample falls just to the west of the range he indicated for that subspecies.

Our data on *H. axyridis* sequence variation are consistent with the hypothesis of a single introduction of that species to eastern North America (Krafsur *et al.* 1997; Lombaert *et al.* 2010), whereas those for *C. septempunctata* suggest multiple introductions from populations with different COI haplotypes (as this species is known to comprise; see Marin *et al.* 2010), a single introduction involving a diverse inoculum, or some combination of the two. Sampling of more populations, including those in their areas of origin in the Old World, would help to unravel the colonization histories of these two species.

The *H. parenthesis* population from wheat in Lamar, Colorado, is notable for comprising just two COI haplotypes, with one of them (Haplotype 9) a singleton in a relatively large sample of 25 animals (Fig. 2). Coccinellids must recolonize annual crops from overwintering sites or other plant formations each season, which, depending upon environmental conditions during the previous season and in diapause (Elliott & Kieckhefer 1990), could lead to severe population depression or local extinction, which in any event is more likely for *H. parenthesis* than the other species because of smaller population sizes (Elliott & Kieckhefer 1990; Elliott *et al.* 1998). Indeed, *H. parenthesis* is less apt to be present continuously in the same localities than other *Hippodamia* species (Elliott *et al.* 1998). Nevertheless, none of the other well-sampled native coccinellid populations sequenced in this study experienced such a pronounced bottleneck. Possible precipitating factors include insecticide use and competition with exotic species. Finally, unlike Springfield and Briggsdale, Lamar is at the edge of this species' range (Gordon 1985); sampling along transects from the edge into the core of the range would clarify whether this has any bearing on the uniqueness of the Lamar *H. parenthesis* population.

In conclusion, our study supports the general utility of COI barcodes for distinguishing and diagnosing coccinellid species, including congeners; however, the method may fail to distinguish recently diverged sibling species

(Fig. 5). In such cases, reciprocal crosses between nominal taxa (e.g. Krafsur & Obrycki 2000; Pérez & Hoy 2002) could be made to assess the presence of reproductive barriers (Mayr 1970). The inclusion of additional morphological, molecular, behavioural and ecological characters, along with distributional data, could also contribute to a fuller understanding of relationships.

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This study reports collaborative research on natural enemy identification, led by Matthew Greenstone. The research was made possible by Natalia Vandenberg's expertise in coleopteran taxonomy and systematics, and Jing Hu's in molecular biology.

Data accessibility

DNA sequences: GenBank accessions JF295977–JF296308.
DNA vouchers: US National Museum Tissue Collection numbers 00530943–00531274.
Morphological vouchers: US National Museum Insect and Mite Collection numbers 00761000–00761331.