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Molecular Evidence From a Parasitoid Wasp, *Schlettererius cinctipes* (Hymenoptera: Stephanidae), for a North American West-to-East Transcontinental Conduit for Wood-Boring Insects

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ABSTRACT Until recently, *Schlettererius cinctipes* (Cresson) (Hymenoptera: Stephanidae) has been restricted to western North America. Since 1996, three specimens of *S. cinctipes* have been found in the eastern United States (Kentucky and Virginia). To determine whether these specimens represent recent introductions from the west, cytochrome *c* oxidase I mitochondrial DNA and one region of 28s rDNA were examined, and pairwise distances were compared. Our results indicate the three specimens represent separate introductions from the west. Because *S. cinctipes* is a parasitoid of wood-boring insects, these conclusions indicate the ease and frequency with which wood-boring insects are transported across the United States.

KEY WORDS recent introduction, invasive species, DNA

Invasion by a non-native species has been recognized as a major environmental problem since the late 1950s and is now regarded as an important consideration in the context of global change (Elton 1958, Vitousek et al. 1996, Sakai et al. 2001). Given the importance of invasive insects, many studies have addressed the environmental effects of introduced insects (Niemelä and Mattson 1996, Pimentel et al. 2000, Nowak et al. 2001, Allen and Humble 2002, Brockerhoff et al. 2006, Haack 2006). Wood-boring invasive species are particularly difficult to control because the most damaging life stages live under bark where damage is not noticed for some time, and topical and foliar insecticides are ineffective (Poland and McCullough 2006, Kreutzweiser et al. 2008). Because control is difficult, it is important to track and prevent the spread of these insects.

The focus of this article is on *Schlettererius cinctipes* (Cresson) (Hymenoptera: Stephanidae). It is not a pest, and thus not invasive, but because it is a parasitoid of wood-boring insects in western North America, it is a clear indicator of the intercontinental transport of its hosts.

S. cinctipes is native to the western United States as well as far west Canada with the eastern-most record in South Dakota (Fig. 1) (Kirk 1975, Aguiar and Johnson 2003). The first record of this species in the eastern United States was of a specimen captured in a suburban back yard in Fairfax Co., VA, in 1996 (Smith 1997). Two more records are reported here for the first time from Kentucky, i.e., 2004 in Harrison Co. and 2007 in Harlan Co. (Fig. 1).

S. cinctipes is an idiobiont primary ectoparasitoid potentially attacking a variety of hymenopterous and coleopterous wood borers (Aguiar and Johnson 2003). *Schlettererius cinctipes* was observed ovipositing into a log infested with high densities of *Monochamus oregonensis* LeConte (Coleoptera: Cerambycidae) and Meyer et al. (1978) suggested *M. oregonensis* could be a host for *S. cinctipes*, but they had no direct evidence. Wood sections used in their research also produced three buprestids and three wood wasps (Hymenoptera: Siricidae) (Meyer et al. 1978). The wood wasps are considered here as potential hosts and are listed as follows: *Urocerus californicus* Norton, *Xeris morrisoni* (Cresson), and *Xeris spectrum* (L). Kirk (1975) observed specimens of *S. cinctipes* emerging from wood infested with the following siricids: *Sirex cyaneus* F., *Sirex juvencus californicus* (Ashmead), *Sirex longicauda* Middlekauff, *Urocerus californicus* Norton, *Xeris morrisoni morrisoni* (Cresson), and *Xeris spectrum* (L.). These species are found only in the western United States, except *S. cyaneus* and *S. longicauda*, which are transcontinental (Schiff et al. 2006).

The only definitive host record for *S. cinctipes* is *Sirex noctilio* F. (Hymenoptera: Siricidae) (Taylor 1967). *S. noctilio* is an exotic species from Eurasia now found in several states in the northeastern United States (Schiff et al. 2006). It was accidently introduced into Australia some time before 1952, where it became a substantial pest in pine (*Pinus* spp.) plantations (Taylor 1976). *S. cinctipes* and several other natural enemies were imported by Australia to control *S. noctilio* (Taylor 1967, 1976; Kirk 1975). Releases were made with specimens from California, Nevada, Ari-

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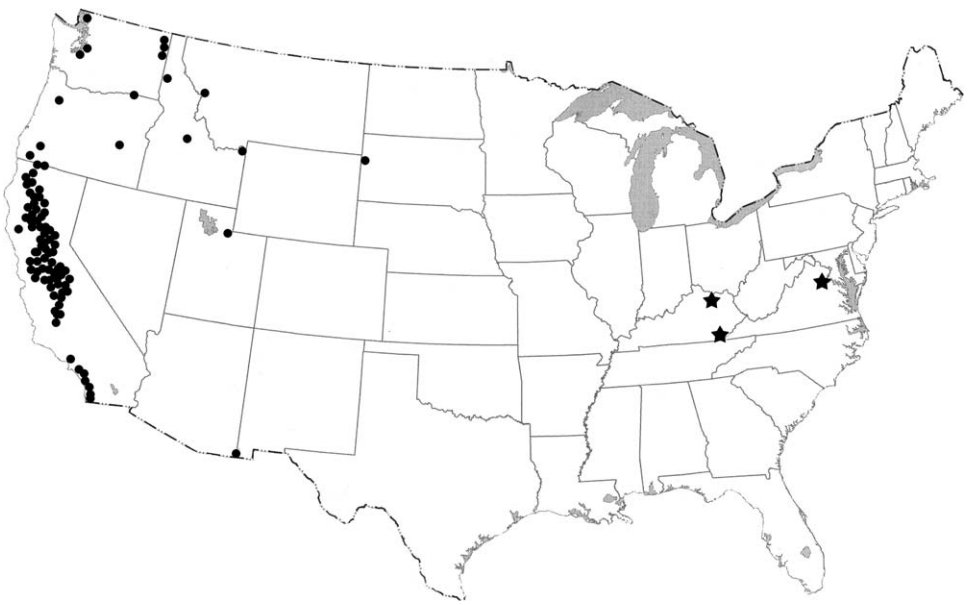

Fig. 1. Distribution of *Schlettererius cinctipes*, modified from Aguiar and Johhson (2003). Dots represent the western, native range of *S. cinctipes*; stars represent new eastern records.

zona, and New Mexico between 1962 and 1973 (Taylor 1976). Any, or all of the above-mentioned species may serve as hosts for *S. cinctipes* and such polyphagy is common for parasitoids of wood-boring insects (Godfray 1994, Hanson and Gauld 2006).

Here, we address several questions. Do the three *S. cinctipes* specimens found in the eastern United States represent an undetected relict population or recent introductions? Because we conclude they are introduced, are they the result of one or multiple introductions? From where were they introduced? When were they introduced? Most importantly, what are the implications for accidental transport of harmful wood-boring insects?

Materials and Methods

Specimen Collection. In addition to the two specimens from Kentucky and one Virginia specimen, four specimens from two locations in California were included. Location, date, and method of collection for all specimens are presented in Table 1. Additional fresh specimens from other localities were unavailable.

Molecular Techniques. Fresh *S. cinctipes* were sequenced for two genes and compared to determine genetic distances. The barcode region of cytochrome *c* oxidase I (COI) mitochondrial DNA as well as domains D1–2 of 28s rDNA were used. COI has a high evolutionary rate and is often used to decipher events at or below the species level (Hurst and Jiggins 2005), but COI also has utility at the population level (Walton et al. 2000) and has even been used to test for multiple introductions of invasive species (Grapputo et al. 2005, Ashton et al. 2008). 28s rDNA was chosen on the basis of ease of amplification and certain regions have been shown to contain enough variation to distinguish cryptic species (Babcock and Heraty 2000, Zhou et al. 2007).

DNA extractions were carried out following the Spin-Column Protocol for Animal Tissues from the DNeasy kit (QIAGEN, Valencia, CA). A leg was removed from each specimen for extraction. Polymerase chain reactions (PCRs) for COI were conducted using a PTC-200 DNA Engine Cycler (Bio-Rad Laboratories, Hercules, CA). All reagents are listed in Table 2. Primer sequences are listed in Table 3. An initial denaturation step was performed at 95.0°C for 2 min 30 s. This was followed by 40 cycles, each consisting of a 94.0°C melting step for 30 s, a 44.0°C annealing step for 30 s, and extension at 68.0°C for 45 s. A final extension was run at 72.0°C for 7 min. All reactions were run with negative controls to detect contamination. PCR prod-

Table 1. Specimen collection

State	Location	Date collected	Collecting method	Specimen
Virginia	Fairfax, Co.; nr. Annandale	4–10 Aug. 1996	Malaise trap	1 female
Kentucky	Harlan Co.; Camp Blanton	4 Aug. 2007	Hand collected	1 female
	Harrison Co.; Silverlake Farm	12–21 July 2004	Malaise trap	1 female
California	San Bernardino Co., Jenks Lake Rd	28 Jan. 2006	Malaise trap	3 females
	Riverside Co.	5 May 2006	Malaise trap	1 female

Table 2. Reagents for PCR and amounts consistent for both COI and 28s reactions (see Table 3 for primer sequences)

Reagent	Concn.	Amt.
Genomic DNA		2 μl
Taq DNA polymerase (New England Biolabs, Ipswich, MA)		0.25 μl
dNTPs	10 μM	3.0 μl
$MgSO_4$	25 μM	2.0 μl
Magnesium-free *Taq* Buffer		2.5 μl
Primers	10 μM	1 μl each

ucts (4.6 μl) were run through 1.0% agarose gels to test the quality of the reaction.

For regions D1-D2 of 28s rDNA, some modifications to the above-described protocol were made. An initial denaturation step at 94.0°C for 2 min 30 s, followed by 35 cycles, each consisting of a 94.0°C melting step for 30 s, a 52.0°C annealing step for 30 s, an extension at 72.0°C for 70 s, and a final extension at 72.0°C for 7 min.

Purification and sequencing of PCR products were performed at the Advanced Genetic Technologies Center (AGTC) at the University of Kentucky (http://www.uky.edu/Centers/AGTC/). Sequences used in this paper are deposited in GenBank as accessions GQ502922 to GQ502934.

Analysis Methods. Sequences were each aligned using MUSCLE (Edgar 2004) and further edited to determine the correct reading frame. All sequences were trimmed to the same length to carry out the pairwise distance calculation.

MEGA 4.1β, downloaded at http://www.megasoftware.net/, was used to calculate the pairwise percent differences between specimens (Tamura et al. 2007, Kumar et al. 2008). The default settings were used and a p-distance calculation, which is the number of base pair differences per site between each sequence, was performed. The p-distance was calculated for all *S. cinctipes* sequences as well two outgroups. Both outgroups are stephanids, i.e., *Neostephanus* sp. JC171 (GenBank accession EF032289) (Schulmeister 2003) for COI; and *Megischus bicolor* (Westwood) (GenBank accession AF379955) (Dowton and Austin 2001) for 28s. These outgroups were chosen to give a frame of reference for the amount of difference among and within species. Unfortunately, exemplars of the other species of *Schlettererius*, *Schlettererius determinatoris* Madl (restricted to Korea), were not available.

Table 3. Primer sequences for all reactions

Primer name	Primer sequence	Reference
CO1lcohym	5′-CAAATCATAAAGATATTGG-3′	Schulmeister et al. (2002)
CO1OutOut	5′-GTAAATATATGRTGDGCTC-3′	Schulmeister et al. (2002)
28SD1shortF	5′-GTGGTAAACTCCATCTAAG-3′	Sharkey lab, unpublished
28SD2shortR	5′-ACATGTTAGACTCCTTGGTC-3′	Sharkey lab, unpublished

Results

The pairwise distance calculated by MEGA is a measure of the number of sites in which two sequences differ. Each pairwise difference, reported on Tables 4 and 5, corresponds to the number of differences divided by the total number of base pairs. A pairwise distance of 0.002 corresponds to a one base pair difference in COI.

The COI pairwise distance analysis data are presented in Table 4, and they correspond to the sequences in Table 6. Only those loci differing among specimens of *S. cinctipies* are shown. COI sequences consisted of 482 bp corresponding to positions 1992–2473 of the *Apis mellifera* L. sequence (Crozier and Crozier 1993). All sequences except those of the outgroup are from *S. cinctipes.* The sequence from S. Australia (GenBank accession EF032237; Schulmeister et al. 2002) is from a specimen collected from Mt. Gambier in 1989.

All specimens from Riverside Co. and San Bernardino Co., CA, have identical COI sequences. There is however one base pair difference between the specimen from South Australia and all California specimens. This difference does not result in an amino acid change. The Harrison Co., KY, specimen is identical to the specimens from San Bernardino Co., CA, and these specimens differ from the Harlan Co., KY, specimen by two base pair differences and infer no amino acid changes. The Fairfax Co., VA, specimen is unique and differs from the S. Australia specimen by one base pair difference.

The 28s pairwise distance calculations are presented in Table 5, with the sequence differences illustrated in Table 7. The portion of 28s used here corresponds to positions 4070–4300 of the *Drosophila melanogaster* (Meigen) sequence (Tautz et al. 1988). For 28s, we were not able to retrieve sequence data for the specimens from Virginia and Riverside, CA. COI and 28s data for South Australia are from the same specimen (GenBank accession AF379957 for 28s) (Dowton and Austin 2001).

As with COI, there are fewer differences between the Harrison Co., KY, specimen and all of the California specimens than there are between the Harlan Co., KY, specimen and the California specimens. There are two base pair differences between the Harrison Co., KY, specimens and the California specimens, and there are five base pair differences separating the Harlan Co., KY, specimen and the California specimens. Unlike the COI sequences, 28s sequences of the California specimens and the Australia specimen are identical. There are seven base pairs that separate the two Kentucky specimens.

Discussion

Introduction(s) or Relict Population? Here, we present arguments for our conclusion that the eastern specimens of *S. cinctipes* represent at least one introduction rather than being the product of a relict population. The first line of evidence comes from collec-

Table 4. Pairwise distance calculations for COI, S. Australia sequence (GenBank accession EF032237)

	Outgroup	San Bernardino Co., CA	Riverside, CA	S. Australia	Harrison Co., KY	Harlan Co., KY
Neostephanus sp., outgroup						
S. cinctipes, San Bernardino Co., CA	0.195					
S. cinctipes, Riverside Co., CA	0.195	0.000				
S. cinctipes, S. Australia	0.197	0.002	0.002			
S. cinctipes, Harrison Co., KY	0.195	0.000	0.000	0.002		
S. cinctipes Harlan Co., KY	0.199	0.004	0.004	0.002	0.004	
S. cinctipes, Fairfax Co., VA	0.195	0.004	0.004	0.002	0.004	0.004

tion data. Aguiar and Johnson (2003) assembled stephanid distribution data from publications and museum specimens. The black dots in Fig. 1 show the collection records for *S. cinctipes* up to 2003. All records are from the western United States. The eastern United States is a very well collected area due to its concentration of museums and universities and it is unlikely that *S. cinctipes* escaped detection if it were there. The map in Fig. 2 shows the distribution of another stephanid, *M. bicolor*, up to 2003 (Aguiar and Johnson 2003), which has been extensively collected in the east. More evidence comes from the Virginia specimen, which was collected from a Malaise trap in 1997. This Malaise trap was run on the same spot since 1981, and this was the first interception of *S. cinctipes* (Smith 1997).

The second line of evidence is from molecular data. Members of a relict population would likely be significantly different from distant populations. Based on COI and 28s sequences, we discovered some eastern representatives of *S. cinctipes* are identical to or very similar to western specimens (see Tables 1–4).

Multiple or Single Introductions? Our pairwise distance data support the hypothesis of multiple introductions. If the eastern specimens were the result of one introduction, we would expect them to be more similar to each other than the California specimens are to each other. There are no base pair differences, for both COI and 28s, between all the California specimens. There are, however, two base pair differences between each of the eastern specimens in the COI sequences. In 28s, there are seven base pair differences between the two included eastern specimens. Our sequence data show the eastern specimens have more differences among them than do the California specimens.

Table 5. Pairwise distance calculations for 28S D1-D2. S. Australia (GenBank accession AF379957)

	Outgroup	San Bernardino Co., CA	S. Australia	Harlan Co., KY
Megischus bicolor, outgroup				
S. cinctipes, San Bernardino Co., CA	0.092			
S. cinctipes, S. Australia	0.092	0.000		
S. cinctipes, Harlan Co., KY	0.107	0.022	0.022	
S. cinctipes, Harrison Co., KY	0.103	0.009	0.009	0.031

Where Are They From? The eastern specimens represent multiple introductions but what are their geographic origins? The Harrison Co., KY, specimen is very similar to the specimens from California. There are no base pair differences in COI, and there are only two base pair differences in 28s. This specimen probably represents an introduction from southeastern California; however it is impossible to confirm its origin without many more samples from a diversity of localities in the western United States. The Harlan Co., KY, specimen has two base pair differences in COI and seven base pair differences in 28s compared with the California specimens. The Virginia specimen has two base pair differences in COI compared with the California specimens and two base pair differences from the Harlan Co. specimen. It is not possible to determine where in the western United States these specimens are introduced from. They could be from a place in California that is very distant from the other specimens or from anywhere else within the native range of *S. cinctipes.* We conclude that all three eastern specimens have independent geographic origins. To determine a precise origin for these specimens, more intensive collecting of *S. cinctipes* needs to take place over its native western range.

When Did They Arrive? It is also not possible to state exactly when these introductions took place. Stephanids have been collected in the east for many years, and it was not until 1996 that a specimen of *S. cinctipes* was intercepted (Smith 1997), and Smith has not captured a specimen since. The eastern specimens probably represent recent introductions although without more information it is impossible to pinpoint how recently they arrived. If more specimens from the west were available, especially those from older collections, it might be possible to determine a more accurate time frame.

Table 6. Base pair differences for COI (base pair positions approximate and not to scale)

	100 bp	200 bp	300 bp	400 bp	500 bp
San Bernardino Co., CA	C	A		C	
Riverside Co., CA	C	A		C	
S. Australia	T	A		C	
Harrison Co., KY	C	A		C	
Harlan Co., KY	T	G		C	
Fairfax Co., VA	T	A		A	

Table 7. Base pair differences for 28s between locations (base pair positions approximate and not to scale)

	50 bp	100 bp		150 bp	200 bp	250 bp
San Bernardino Co., CA	C	G	C	C	T	A
S. Australia	C	G	C	C	T	A
Harlan Co., KY	A	C	T	T	T	A
Harrison Co., KY	C	G	C	C	G	T

What Are the Environmental and Economic Implications? The conclusion that there have been multiple introductions of *S. cinctipes* from the west is cause for alarm. The specimens were almost certainly transported with wood-boring hosts from the west, and these hosts could potentially be major pests not yet introduced to the eastern United States.

The case of *S. cinctipes* illustrates a novel, although indirect way to track wood-boring insects that could have devastating consequences in areas outside of their native range. In their native range, wood-boring species often are not harmful to their environment. Outside of their native range, however, wood-boring insects have the potential to be very destructive (Taylor 1976; Kucera 1996; Haack et al. 1997, 2002; Haack 2006). Based on this study, we cannot be sure what wood-boring insect *S. cinctipes* was using, but siricid larvae seem the most plausible. Siricids native to the eastern United States would not be cause for alarm. However, if it was one of the several western endemic species (i.e., *U. californicus*, *X. morrisoni morrisoni*, or *S. juvencus californicus*), this could be a large problem because the effect these species could have on eastern U.S. ecosystems is unknown (Schiff et al. 2006). These species all feed on conifers, mostly Pinaceae (Schiff et al. 2006), and could all be harmful, but of particular concern is *U. californicus.* This species feeds on hemlock (*Tsuga* spp.), and in the eastern United States whole stands of hemlock are currently threatened by an invasive pest, the hemlock wholly adelgid, *Adelges tsugae* (Annand). More pests would exacerbate this problem (Orwig et al. 2002). Currently, there is a U.S. *Sirex* survey (NAPIS 2008) focusing on new locality data for *S. noctilio*, which has become a major pest in Australia and has potential to do the same in the eastern United States. There have been several reports of *S. noctilio* in the eastern United States in recent years (Long et al. 2009) and because it is a host of *S. cinctipes*, their recent co-occurrence in the east may not be coincidental. The survey may do well to search for *S. noctilio* in habitats where *S. cinctipes* has been found in the east.

Wood-boring insects can be detrimental to orchards, destroy entire forests and urban landscapes, and be difficult to control by chemical means (Poland and McCullough 2006, Kreutzweiser et al. 2008). The current study elucidates the ease at which wood-boring insects can be transported beyond their native range without detection and the need to pay attention to wood transport across the continent.

Numerous studies have been conducted and regulations exist to prevent invasive species transportation between countries (Anonymous 1994, Jenkins 1996, Mack et al. 2000). Although quarantine measures exist that are designed to prevent the spread of invasive species, little is done to prevent indigenous species with restricted distributions from being transported. Given that within one country there are many different habitat types, with very diverse and different flora and fauna, it seems prudent to regulate the spread of native species beyond their present geographic ranges.

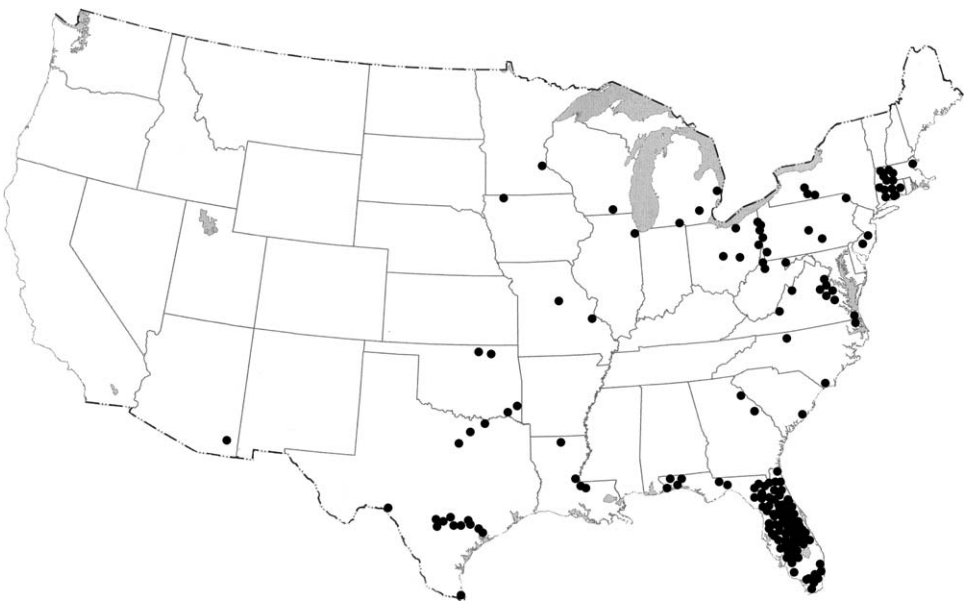

Fig. 2. Distribution of *Megischus bicolor*, modified from Aguiar and Johhson (2003).

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