Journal of Entomology, 2010
ISSN 1812-5670

Identification of *Sirex noctilio* and Native North American Woodwasp Larvae using DNA Barcode

A.D. Wilson and N.M. Schiff
USDA Forest Service, Center for Bottomland Hardwoods Research,
Southern Hardwoods Laboratory, P.O. Box 227, Stoneville, MS 38776-0227, USA

Abstract: The exotic Sirex woodwasp (*Sirex noctilio*), recently introduced into native pine forests of the northeastern US and Canada, will likely become a major pest of pines and possibly other conifers in North America. This eurasian siricid woodwasp causes damage by the injection of a phytotoxic mucus and inoculum of a damaging exotic wood decay fungus (*Amylostereum areolatum*) into living pine trees at oviposition. Larvae feed on pine wood decayed by the fungus and on the fungus itself, producing large galleries in the wood. *S. noctilio* larvae are morphologically indistinguishable from endemic North American siricid larvae. The aim of this study was to develop a diagnostic molecular method for identifying native and nonnative siricoid woodwasps in North America. A modified DNA barcode method was tested as a means for identifying and distinguishing *S. noctilio* larvae from native woodwasp larvae in wood based on PCR amplification and sequencing of a 680-basepair region of the mitochondrial gene cytochrome c oxidase I (CO1). Comparison of DNA homology within the barcode region between *S. noctilio* and 22 native woodwasps showed numerous locations in this gene where differences in base substitutions were detected, allowing effective identification and discrimination from all North American woodwasps tested. These methods also are useful for identifying nonnative woodwasp larvae detected or encountered in wood during wood-importation inspections at US and Canada ports of entry, in forest exotic insect-pest surveys and for measuring taxonomic relatedness between woodwasp species. The woodwasp-specific, CO1-based DNA barcode method developed here has proven to be a reliable and efficient new tool useful for the rapid and accurate identification of siricoid woodwasp larvae and adults. The significance and possible limitations of DNA barcode methods for biosecurity applications are discussed.

Key words: Cytochrome c oxidase I gene, sequence homology, siricoidea, wood borers

INTRODUCTION

Recent introductions of the invasive exotic Sirex woodwasp, *Sirex noctilio* F. (Hymenoptera: Siricidae), into pine forest stands near Oswego New York in the United States and southeastern Ontario in Canada in 2004 and 2005 are a serious concern to forest pest

Corresponding Author: A.D. Wilson, USDA Forest Service,
Center for Bottomland Hardwoods Research,
Southern Hardwoods Laboratory, P.O. Box 227, Stoneville,
MS 38776-0227, USA

managers in North America (Hoebeke *et al*., 2005; Haugen and Hoebeke, 2005; Yemshanov *et al*., 2008, 2009). *Sirex noctilio* is native to Eurasia where it is not a pest, but it has had a major economic impact on pines (*Pinus* sp.) where introduced into the Southern hemisphere countries of New Zealand, Australia, South America and South Africa; devasting millions of hectares of native and exotic pine forests in the absence of its natural biocontrol agent, the parasitic nematode *Deladenus siricidicola* (= *Beddingia siricidicola*) (Hurley *et al*., 2007). This siricid woodwasp is now an important alien invasive pest of North American pines and a serious threat particularly to species planted in the southern United States. It causes damage by injecting a phytotoxic mucus and inoculum of a damaging wood decay fungus (*Amylostereum areolatum*) into living pine trees at oviposition and the production of galleries by larvae feeding in both sapwood and heartwood (Wilson *et al*., 2009). *Amylostereum areolatum* is a mycosymbiont carried internally in posterior abdominal glands (mycangia) of adult female woodwasps that effectively vector the fungus to its tree hosts. Adult *S. noctilio* females are capable of flying more than 80 km in a single season. Arthrospores or hyphal fragments (oidia) of *A. areolatum*, stored in the mycangia, are introduced into the sapwood of uninfested trees via the oviduct and the ovipositor that penetrates deeply through pine bark. Mycosymbionts of xiphydriid and siricid woodwasps cause considerable damage through discoloration and white-rot type decay of host woods (Wilson and Schiff, 2003; Wilson *et al*., 2004).

The inability to recognize *S. noctilio* larvae in native pine woods recently has become a major problem in North America because this species is morphologically indistinguishable from native North American siricid larvae. The ability to identify *S. noctilio* larvae in wood during pest-detection surveys of native forests is essential in order to implement timely pest-control measures for this exotic woodwasp in newly-infested forest stands before a reproductive population becomes well established. The ability to identify *S. noctilio* larvae in imported woods, including raw logs, processed lumber, packing crates and even wooden furniture, is equally important during inspections of imported woods at US ports of entry to prevent new introductions of this pest into locations with susceptible tree hosts. DNA barcode technology (Hebert *et al*., 2003), based on sequencing of the cytochrome oxidase gene (CO1), is particularly well suited in biosecurity for invasive species identification (Armstrong and Ball, 2005). Among molecular diagnostic technologies used in early detection systems to identify invasive nonnative species, DNA barcode methodologies have been used successfully for the identification of many insects including some of the most economically significant global pests such as the tephritid fruit flies (Armstrong *et al*., 1997), lymantriid tussock moths (Armstrong *et al*., 2003), tortricid leafroller moths (Dugdale *et al*., 2005), various thrips (Toda and Komazaki, 2002) and morphologically-similar species such as the tropical tachinid parasitoid flies (Smith *et al*., 2007).

Invasive exotic forest insect pests threaten the stability of forest ecosystems, the livelihoods of industrial producers (especially within the forest-products industry) and consequently affect consumer confidence via price fluctuations, potentially resulting in serious economic impacts due to ripple-effects on many dependent industries (Cock *et al*., 2003; Williamson, 1996). These nonindigenous invasive tree pests also have dramatically affected the diversity, productivity and functions of forested ecosystems throughout North America (Mack *et al*., 2000; Pimentel *et al*., 2000; McCullough *et al*., 2006). The high economic risks associated with introductions of invasive alien insects mandate the development of effective and efficient methods for the rapid identification of these exotic plant pests found in forest-monitoring surveys or intercepted in infested imported woods at major US ports of entry. We received requests from officials within the USDA Animal and

Plant Health Inspection Service (APHIS), Plant Protection and Quarantine program, to develop molecular methods for the identification of *S. noctilio* larvae in wood that would also provide a means for identifying native woodwasp larvae found in imported woods and in forest insect surveys. Consequently, the objectives of this research were to 1) develop and test a modified DNA barcode method specific for woodwasps (Siricoidea) to identify and discriminate alien or nonindigenous *S. noctilio* larvae from larvae of twenty-two native North American woodwasp species found in infested wood, based on PCR amplification and sequencing of a 650-700 basepair region of the cytochrome c oxidase I mitochondrial gene (CO1) and 2) to analyze the sequence homologies of native woodwasps compared with *S. noctilio* using DNA sequence data from the CO1 barcode region. Some preliminary results of this research were reported previously (Wilson and Schiff, 2006).

MATERIALS AND METHODS

The present research study was conducted during a three-year period from 15 June 2005 to 30 September 2008, concurrent with a Special Technology Development Program (STDP) grant received from the USDA Forest Service, Forest Health Protection (FHP), through the Southern Research Station (SRS). All of the research materials and samples were prepared and processed in research laboratories of the USDA Forest Service (FS), Southern Hardwood Laboratory and in USDA Agricultural Research Service (ARS) quarantine and molecular biology laboratory facilities in Stoneville, Mississippi, USA.

Collection of Wood Samples Containing Woodwasp Larvae

Wood infested with woodwasp larvae of major North American Siricoid taxa were collected and reared from conifer and hardwood logs throughout the United States and Canada. The number of individuals sequenced per woodwasp species depended on availability of infested wood and geographic distribution of specimens. Bole sections of infested stems were collected from trees of the following species: *Abies amabilis* Douglas ex Forbes, *Acer saccharum* Marsh., *Betula nigra* L., *Betula papyrifera* Marsh., *Calocedrus decurrens* (Torr.) Florin, *Carpinus caroliniana* Walter, *Celtis laevigata* Willd., *Fraxinus pennsylvanica* Marsh., *Juniperus occidentalis* Hook., *Pinus banksiana* Lamb., *Pinus contorta* Loudon, *Pinus ponderosa* Douglas ex Lawson and C. Lawson, *Pinus radiata* D. Don, *Pinus resinosa* Aiton, *Pinus sylvestris* L., *Pseudotsuga menziesii* (Mirb.) Franco, *Quercus phellos* L., *Taxodium distichum* (L.) Rich., *Tilia americana* L. and *Tsuga mertensiana* (Bong.) Carrière. Conifer and hardwood logs and boles infested with siricid woodwasp larvae often were recognized externally by the presence of ovipositing females or the very long ovipositor-remains of *Megarhyssa* sp. parasitoids sticking out perpendicular to the surfaces of infested boles. Hardwood limbs infested with xiphydriid larvae usually were discovered by the presence of zone lines, seen at the ends of broken limbs, that were produced in xiphydriid-infested host woods by the ascomycetous wood-decaying mycosymbionts (*Daldinia* sp., *Xylaria* sp.) of these woodwasps (Wilson and Schiff, 2000a, b; Wilson *et al.*, 2004). Log sections were cut into bolts up to 90 cm long in the field and shipped back to the lab for processing. Some of the log sections were split and dissected with an axe, hatchet, or splitting maul (for small limbs) to collect representative woodwasp larvae in the field. Larvae were placed into 95% ethanol in glass screw-capped vials with collection data labels inserted. A few of the remaining unprocessed logs were placed into plastic trash cans fitted with a screen window in the lid for moisture ventilation. A hole was cut into the side of the can into which a large glass jar was inserted to capture

emerging adults. Alternatively, log bolts were placed into large terrariums precisely fitted with a plywood board cover containing a handle for rapid access. *Sirex noctilio*-infested red pine bole sections were reared in large plastic containers within the receiving isolation room of the USDA-ARS Bldg. 8 quarantine facility in Stoneville, MS. Emerging adults, serving as voucher specimens for each site collection, were captured by hand or using butterfly nets as needed. Adults were preserved in 95% ethanol for subsequent total DNA extractions.

Known reference specimens of adult woodwasps, useful for species confirmations, were obtained from various external sources and collectors in many countries. Additional unidentified woodwasp larvae were obtained through USDA-APHIS interceptions of insects collected from imported woody samples that had been examined over many years by quarantine inspectors and identifiers at various ports of entry in the United States. Other sources of unidentified woodwasp larvae were obtained from collectors and taxonomist at various US government institutions and agencies and by insect trapping and wood collections by US Forest Service employees.

Isolation of Total DNA from Woodwasps

The methods used for isolation of total DNA from woodwasp larvae and adults followed the general procedures for DNA barcode analyses described by Hebert *et al.* (2003) with some modifications as follows. Insect specimens stored at -80°C or in 95% ethanol were removed from labelled collection vials and air-dried to remove any residual ethanol. Sterile forceps were used to remove a single thoracic leg from larvae or adults of each woodwasp specimen for total DNA isolation using a Qiagen DNeasy Mini Spin column kit (Qiagen, Valencia, CA). Leg tissue, removed from where it was attached, was place in an autoclaved 2 mL microtube labeled with sample number, covered with 180 µL Qiagen tissue lysis Buffer ATL and ground in the tube using a Konte pestle. Ground tissue was mixed immediately and thoroughly by vortexing with 20 µL proteinase K, incubated at 55°C in a heating block (containing distilled water) for 1-3 h with occasional vortexing during incubation to disperse sample until cells of the sample tissue were completely lysed. Each sample solution was again vortexed for 15 sec, amended with 200 µL Buffer AL, mixed thoroughly and incubated at 70°C for 10 min. Ethanol precipitation of total sample DNA was achieved by adding 200 µL ethanol (96-100%) to the sample, mixing thoroughly by vortexing and pipetting the mixture with any precipitate into a DNeasy Mini spin column (Qiagen) placed into a 2 mL collection tube, centrifuged at 8,000 rpm for 1 min and discarding the flow-through effluent and collection tube. The DNeasy Mini spin column was placed into a new 2 mL collection tube to which 500 µL Qiagen wash Buffer AW1 was added, centrifuge at 8,000 rpm for 1 min and again the flow-through effluent was discarded with the collection tube. This step was repeated again with a new 2 mL collection tube with 500 µL of Qiagen wash Buffer AW2 and centrifuged 3 min at 14,000 rpm to dry the DNeasy membrane and discarding the flow-through effluent and collection tube to elute and remove all residual ethanol. The DNeasy Mini spin column was placed into a new collection tube to which 200 µL Ambion nuclease-free H_2O (Applied Biosystems, Foster City, CA), was added directly onto the DNeasy membrane, incubated at 21°C for 1 min and centrifuge for 1 min at 8,000 rpm to elute the DNA product flow-through effluent. This final product was pipetted into a final 2 mL microtube, labeled with the sample tube number and stored at -20°C until PCR amplification.

PCR Amplification of CO1 Gene and Gel Electrophoresis of PCR Products

We utilized PCR to amplify the DNA Barcode region (CO1) of woodwasp mitochondrial DNA. Appropriate amounts of total insect DNA sample product from each extraction were

utilized in PCR reactions with primers LCO 1490 and HCO 2198. The master mix reaction was prepared by combining 19 μL of Ambion nuclease-free water with 2.5 μL of Advantage 2 10X PCR buffer, 1 μL each of HCO 2198 and LCO 1490 primers, 0.5-0.75 μL dNTPs (10 μM). The PCR reaction mixture, prepared by dilution with Ambion nuclease-free H_2O and Advantage 2 PCR buffer, consisted of a final reaction concentration of 40 mM tricine-KOH (pH 8.7 at 25°C), 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 μg mL^{-1} BSA, 0.005% v/v Tween 20, 0.005% v/v nonionic detergent P40, 10 mM of each of the four dNTPs, 10 μM of each of the two primers and 0.4 μL of high fidelity Advantage 2 Taq polymerase (ClonTech, Mountain View, CA) per reaction. Extracted genomic DNA (5-10 ng) was used as template for the amplification reactions.

PCR reactions were performed on a PTC-100 PCR machine (MJ Research, Inc., Waltham, MA) using a protocol consisting of forty-one cycles of 95°C for 30 sec, primer annealing at 45°C for 60 sec and elongation at 72°C for 60 sec, followed by one final cycle of elongation at 72°C for 30 min, ended by cooling at 4°C. Ultra pure Ambion nuclease-free water was used as a PCR control. A sample of the PCR products was subjected to electrophoresis on 7.5% acrylamide gels run for 35-40 min at 200 v using a Mini-Protean 3-gel vertical electrophoresis system (Bio-Rad, Hercules, CA). The gel was stained with 1% (w/v) ethidium bromide and optimally destained in distilled water. PCR products in the gel were subsequently visualized by UV illumination with Fotodyne Foto/prep UV light box and quantified by band-color intensity compared with a low DNA mass ladder (Introgen, Carlsbad, CA) of 100-2000 bp containing know quantities (10-200 ng) of DNA. Final DNA preparations were stored at -20°C.

DNA Sequencing and Sequence Data Analysis

Molecular identifications were achieved by PCR amplification of CO1 products from total DNA sample extractions that were directly sequenced in both directions. PCR products were purified prior to sequencing by adding 20 μL of PCR product (20 ng μL^{-1}) to 2.0 μL shrimp alkaline phosphatase (SAP) and 0.2 μL Exonuclease I (EX-I) in a microtube with filter tip and subjected to a thermocycler run using a program consisting of a single cycle of 37°C for 1 h, 80°C for 15 min and cooling to 4°C. All purified PCR products were stored at -20°C until sequenced. DNA sequencing of the purified amplified CO1 products were performed using a 3730xL DNA Analyzer automated DNA sequencer (Applied Biosystems, Foster City, CA). CO1 gene sequences from replicate woodwasp samples were inspected, corrected for misreads and gaps and aligned with SeqManII software (DNASTAR Inc., Madison, WI). Concensus sequences of woodwasp samples were subjected to BLAST program analyses to compare against the NCBI GenBank database to determine sequence homology with existing GenBank sequences.

RESULTS

Collection of Wood Samples Containing Woodwasp Larvae

Woodwasp larvae of all major North American siricoid families were reared from infested conifer and hardwood logs collected at many locations from host trees in the United States, Canada and miscellaneous countries throughout of the world (Table 1). Woodwasp larvae collected from split bole sections were highly variable in size and developmental stages. The smallest larvae came from xiphydriid, orussid and syntexid specimens although some siricid larvae were surprisingly small when collected from xeric environments. The larger siricid larvae also were highly variable is size, but generally much larger than larvae of the other

Table 1: Woodwasp collections and associated woody hosts of North American and non-native woodwasp larvae utilized in sequence homology comparisons of the CO1 gene

Woodwasp species	Collection sites[1]	County, State, or Country	Tree hosts[2]	Host common name
Siricidae				
Eriotremex formosanus	Delta N.F.	Sharkey Co., MS, LA	*Quercus phellos*	Willow oak
	Noxubee N.W.R.	Oktibbeha Co., MS	*Carpinus caroliniana*	American hornbeam
	Angelina N.F.	Angelina Co., TX	*Fraxinus pennsylvanica*	Green ash
Tremex columba	Delta Exp. For.	Washington Co., MS, NY	*Celtis laevigata*	sugarberry
Sirex cyaneus		AK, CA, CO, MT, WA	Unknown	
	Willamette N.F.	Jefferson Co., OR	*Pinus* sp.	Western pine
	S. fork Sprague R.	Klamath Co., OR	*Pinus ponderosa*	Ponderosa pine
Sirex edwardsii		IN, LA, MS, NY	Unknown	
Sirex juvencus californicus	El Dorado N.F.	CA, MT, OR, WA	*Calocedrus decurrens*	Incense-cedar
Sirex juvencus juvencus		Greece, Italy, Spain	Unknown	
Sirex nigricornis		IN, NY, LA	Unknown	
Sirex noctilio	Tasmania	Australia	*Pinus radiata*	Monterey pine
		Argentina	*Pinus banksiana*	Jack pine
		Oswego Co., NY	*Pinus resinosa*	Red pine
			Pinus sylvestris	Scotch pine
Urocerus albicornis		Nova Scotia, NY, WA	Unknown	
Urocerus californicus	Shasta N.F.	Shasta Co., CA, OR, WA	*Pseudotsuga menziesii*	Douglas fir
Urocerus cressoni		CO, IL, IN, Nova Scotia, NY	Unknown	
Urocerus gigas flavicornis		AK, CA, WA	Unknown	
	S. fork Sprague R.	Klamath Co., OR	*Pinus ponderosa*	Ponderosa pine
			Pinus contorta	Lodgepole pine
Urocerus taxodii	Delta N.F.	MS	*Taxodium distichum*	Bald cypress
Xeris morrisoni indecisus	Deschutes N.F.	Deschutes Co., OR	*Abies amabilis*	Pacific silver fir
			Tsuga mertensiana	Mountain hemlock
Xeris spectrum spectrum		Nova Scotia, WA	Unknown	
Xeris spectrum townesi		WA	Unknown	
Xiphydriidae				
Xiphydria abdominalis	Brownfield woods	Champaign Co., IL	*Tilia americana*	American basswood
Xiphydria decem	Roadside	Pope Co., IL	*Betula nigra*	River birch
Xiphydria maculata	Dixon Sprs. Res.Cen.	Pope Co., IL	*Acer saccharum*	Sugar maple
	Brownfield woods	Champaign Co., IL	*Acer saccharum*	Sugar maple
			Tilia americana	American basswood
	Forest Glen. Pres.	Vermilion Co., IL	*Acer saccharum*	Sugar maple
Xiphydria mellipes	High Cliff St. Pk.	Calumet Co., WI	*Betula papyrifera*	Paper birch
Xiphydria scafa	Corning	Randolf Co., AR	*Carpinus caroliniana*	American hornbeam
Xiphydria tibialis	Forest Glen. Pres.	Vermilion Co., IL	*Acer saccharum*	Sugar maple
	Corning	Randolf Co., AR	*Carpinus caroliniana*	American hornbeam
	Brownfield woods	Champaign Co., IL	*Carpinus caroliniana*	American hornbeam
Orussidae				
Orussus occidentalis		El Dorado Co., CA	Unknown	
Orussus terminalis		NY	Unknown	
Orussus thoracicus		CA	Unknown	
Anaxyelidae				
Syntexis libocedrii	Klamath Falls	Klamath Co., OR	*Juniperus occidentalis*	Western juniper
			Pinus ponderosa	Ponderosa pine
	Shasta N.F.	Shasta Co., CA	*Pseudotsuga menziesii*	Douglas fir

[1]Locale of the collection sites within the region (county, state, or country) indicated for each woodwasp collection. [2]Tree hosts usually indicate the wood type from which larvae were extracted or from which adults were reared. In a few cases, these were the tree types from which adults were collected, but not necessarily the true hosts from which the adults emerged. Some adult specimens were caught on the wing and no host association could be determined (labelled unknown)

taxa. All woodwasp specimens contained the characteristic posterior spine (postcornus), a protrusion that is diagnostic of these specialized hymenopterous wood borers.

Specimens of *Sirex noctilio* adults and larvae were collected from red pine (*Pinus resinosa* Aiton) bole sections taken from an infested pine stand in Oswego Co., New York State. The symptomology and damage associated with attacks by this exotic pest were considerably more dramatic than damage associated with infestations by native North American woodwasps. The severity of visible symptoms in affected trees, (including needle wilting, leaf necrosis, resinosis and vascular discoloration), were largely attributed to the production and injection of a mucus toxin into affected trees, along with inoculum (hyphal fragments or oidia) of the mycosymbiont *Amylostereum areolatum* and eggs by adult females at oviposition (Fig. 1 a-c). Round emergence holes of adults ranged from 3-6 mm in diameter and tended to occur in clusters on the bole (Fig. 1d). Adult *S. noctilio* females were black and

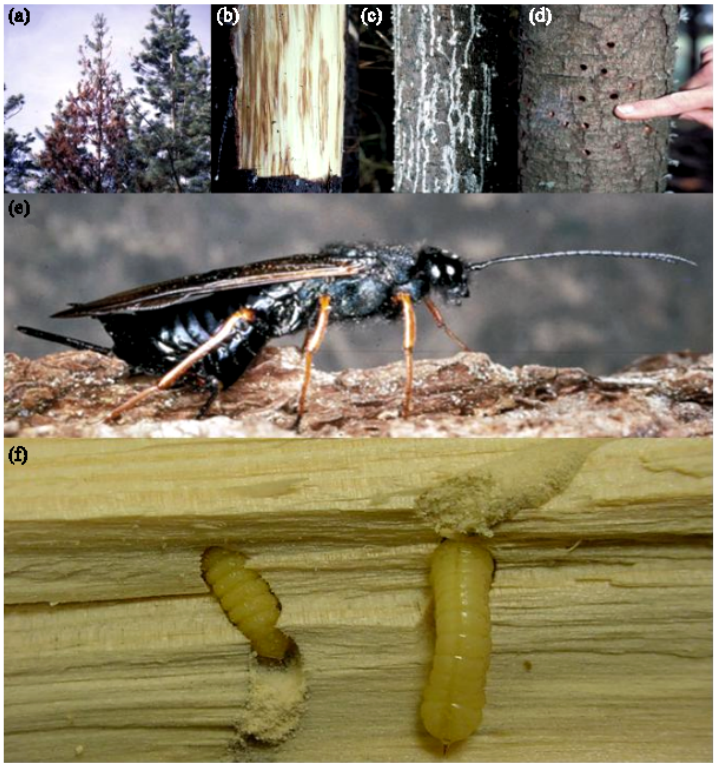

Fig. 1: External and internal damage cause by exotic woodwasp pest *Sirex noctilio* in North American pines. (a) Needle wilting and necrosis caused by translocated mucus toxin; (b) Vascular discoloration (staining) in response to wood-decaying mycosymbiont *Amylostereum areolatum*; (c) Leaking of pine resin (resinosis) from a *S. noctilio*-infested bole; (d) Adult emergence holes on bole; (e) Adult female of *S. noctilio* collected from red pine (*Pinus resinosa*) in Oswego, Co., New York State; (f) S-shaped larvae of *S. noctilio* in red pine log galleries, tightly packed with frass and adjacent wood with incipient wood decay damage; notice that *S. noctilio* larvae have a postcornus (posterior spine) protruding from the end of the abdomen like other woodwasps, but are morphologically indistinguishable from native siricid woodwasp larvae

generally quite similar in appearance to other black North American siricid females such as *S. cyaneus*, *S. edwardsii* and *S. varipes* (Fig. 1e). Adults of these species are morphologically distinguishable only by closer examination of stereoscopic characters. Larvae of *S. noctilio* are cream-colored and currently not morphologically distinguishable from the larvae of native siricid woodwasps (Fig. 1f). They produced an abundance of frass in galleries not unlike other siricid larvae. Several galleries containing *S. noctilio* larvae had an unidentified blue-stain fungus emanating from them that was growing and colonizing sapwood directly above each gallery. Incipient wood decay by the mycosymbiont *A. areolatum* was detected around and between adjacent galleries by the presence of slightly punky and softened wood and the whitening of cellulose in these areas indicating the partial removal of lignin diagnostic of white-rot type decay.

Isolation of Total DNA from Woodwasps

We were unable to extract undegraded DNA from specimens of *Xiphydria abdominalis*, *X. decem* and *X. scafa* due to various problems associated with preservation, extraction and contamination. The condition and freshness of the specimens as well as storage solutions utilized for specimen preservation prior to DNA extraction varied somewhat with difference sources and collectors. Undegraded DNA was extracted from specimens frozen at -80°C and stored in 96-100% ethanol prior to extraction.

PCR of CO1 Gene and Gel Electrophoresis of PCR Products

PCR amplification of the mitochondrial gene cytochrome c oxidase I (CO1) of woodwasps, using the aforementioned thermocycler program, yielded approximately 15-30 ng μL^{-1} of total DNA in each PCR product. DNA sequencing of PCR products yielding at least 20 ng μL^{-1} of DNA were most successful. All PCR-amplifications from woodwasps larvae and adults yielded a single product of good purity in the range of 560-700 bp for the woodwasps tested.

A comparison of the amplified CO1 gene product of exotic woodwasp pest (*Sirex noctilio*) with various native North American woodwasps indicated the relative size of the CO1 product obtained for each species (Fig. 2). Among the species compared here, the largest CO1 amplified products were obtained for *Orussus thoracicus* and *Urocerus taxodii* woodwasps (about 850 bp) and the smallest products were obtained for *Xiphydria mellipes* and *Xiphydria tibialis* woodwasps (about 750 bp), although only about 650-700 bp within these product sequences were useful for discrimination of most woodwasp species. No double or multiple bands were observed among CO1 gene products for any woodwasp species indicating that only a single pure product was amplified in each case.

DNA Sequencing and Sequence Analyses

An adult female woodwasp collected in March 2005 from a Fulton, New York exotic beetle survey trap was identified as the exotic pest *Sirex noctilio*, or European woodwasp. This was a recent confirmed indication that this pest had been introduced into the United Status. A ground search near Oswego, NY located five infested red pine (*Pinus resinosa*) trees containing siricid larvae. Additional larvae were found in infested scotch pine (*Pinus sylvestris*) trees at another nearby site in New York State. The CO1 barcode gene sequences of the unknown larvae were compared with sequences of identified adult woodwasps subsequently reared from the same bole sections. The sequences of four unknown larvae from two different sites in New York were exact matches to the sequence of *S. noctilio* adults from Tasmania, Australia. The sequence of the unknowns differed from all native North American siricoid woodwasps tested. Three months later, adult *S. noctilio*

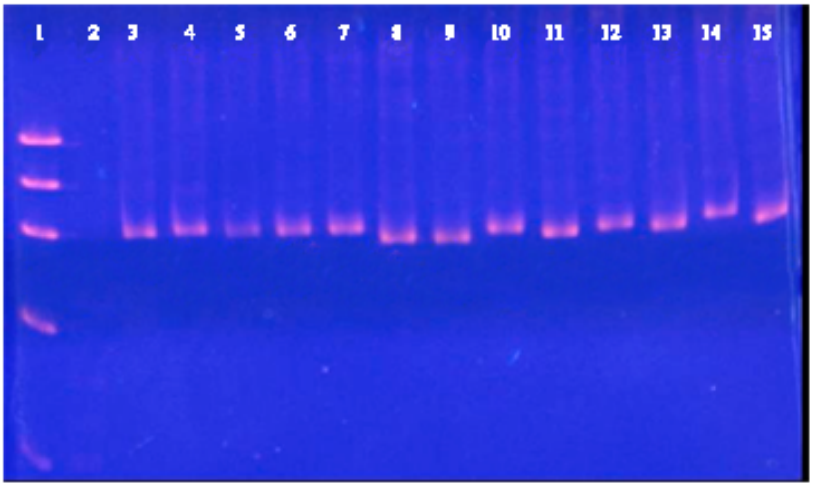

Fig. 2: PCR-amplified products from the 650-700 base pair region of the mitochondrial gene cytochrome c oxidase I (CO1) of exotic woodwasp pest (*Sirex noctilio*) and various native North American woodwasps using LCO 1490 and HCO 2198 primers. Lane 1 is low molecular weight ladder (ldr) consisting of 200-2,000 bp (range from bottom to top); lane 2 ultrapure water control; lane 3 and 13 from *Xiphydria abdominalis* (XA); lane 4 from *Xiphydria maculata* (XMAC); lane 5-7 from exotic siricid *Sirex noctilio* (SN); lane 8 from *Xiphydria mellipes* (XMEL); lane 9 from *Xiphydria tibialis* (XT); lane 10 from *Sirex edwardsii* (SE); lane 11 from *Sirex nigricornis* (SNG); lane 12 from *Xeris morrisoni* (XEM); lane 14 from *Orussus thoracicus* (OT); lane 15 from *Urocerus taxodii* (UT)

specimens emerged from bole sections collected from infested trees that were held in quarantine. DNA barcode sequences of these adults reared from the same logs from which larvae were collected confirmed the larval determinations. The large number of differences between related and distant siricoid taxa and the exact match of unknowns to identified Tasmanian *S. noctilio* specimens left no doubt that the unknown New York specimens were *S. noctilio*. The identification of larval infestations in resident trees confirmed that the introduction of *S. noctilio* into the US was successful. Subsequent trapping of adult female *S. noctilio* specimens up to 80 km from the original infested trees indicated that this pest was rapidly spreading. Other reports of *S. noctilio* adults were found at several locations across Lake Ontario in stands near the north shore in Ontario, Canada.

Barcode sequences were produced for 207 individual woodwasps specimens, consisting of 21 species from 4 families and 5 of 6 subfamilies (except Orussidae: Ophrynopinae) of native North American Siricoidea and 29 specimens of two non-native siricids, *S. noctilio* from Australia (Tasmania) and Argentina and *S. juvencus juvencus* from Europe (Greece, Italy and Spain). The differences in barcode sequences for all native woodwasp species were compared in terms of base substitutions relative to the sequences of *Sirex noctilio* specimens (the exotic pest) and percent differences in bases substitutions or sequence homologies were determined (Table 2). Native North American woodwasps of the genus *Sirex* were most closely related to *S. noctilio*, differing in base substitutions from this exotic siricid by only 50-73 bases or 7-11%, respectively. *Sirex noctilio* differed by only 50 of

Table 2: Differences in sequence homology of twenty-two North American and non-native woodwasps relative to the exotic pest *Sirex noctilio* using DNA barcode

Family	Woodwasp species	n	Substitutions	Mean % difference[1]
Siricidae				
Tremecinae	*Eriotremex formosanus*	4	137/696	19.7
	Tremex columba	5	119/635	18.7
Siricinae	*Sirex cyaneus*	16	58/694	8.4
	Sirex edwardsii	13	68/691	9.8
	Sirex juvencus californicus	8	73/693	10.5
	Sirex juvencus juvencus	6	50/656	7.6
	Sirex nigricornis	15	58/560	10.4
	Sirex noctilio	23	0/698	0.0
	Urocerus albicornis	7	111/687	16.2
	Urocerus californicus	12	116/694	16.7
	Urocerus cressoni	8	120/695	17.3
	Urocerus gigas flavicornis	10	106/695	15.2
	Urocerus taxodii	6	123/694	17.7
	Xeris morrisoni	6	126/560	22.5
	Xeris spectrum spectrum	10	138/694	19.9
	Xeris spectrum townesi	7	123/700	17.6
Xiphydriidae	*Xiphydria maculata*	8	154/698	22.1
	Xiphydria mellipes	5	147/590	24.9
	Xiphydria tibialis	7	143/590	24.3
Orussidae	*Orussus occidentalis*	11	169/691	24.4
	Orussus terminalis	2	164/626	26.2
	Orussus thoracicus	4	161/698	23.1
Anaxyelidae	*Syntexis libocedrii*	14	173/699	24.7

[1]Percent differences in sequence homology of CO1 gene relative to exotic pest *Sirex noctilio* based on nucleobase substitutions

656 base substitutions or 7.6% from *Sirex juvencus*, a European congener that is adventive in the United States. This was the smallest difference found among all woodwasps examined. Both of these species, *S. noctilio* and *S. juvencus juvencus*, are native to Europe and are not known as pests due to natural biocontrol by endemic parasitic nematodos. The small genetic difference in barcode homology among siricid species also was correlated with morphological similarities between the non-native *S. noctilio* and several black native species such as *S. cyaneus*, *S. edwardsii* and *S. varipes* that closely resemble this pest. Base-substitution differences between *S. noctilio* and *S. cyaneus* at positions 218, 224, 236, 241, 254 and 260 are indicated within a 10% representative segment of the CO1-gene sequence (Fig. 3). Although significant interspecific differences in base substitutions were readily obtained, no intraspecific differences were found in any species tested. Overall, the ranking of percent homological differences relative to *S. noctilio* was greatest in the Orussidae, less in the Xiphydriidae and Anaxyelidae and least in the Siricidae.

Comparisons of CO1 gene base-substitution differences relative to *S. noctilio* for subspecies of *Sirex juvencus* and *Xeris spectrum* indicated relatively large percentage differences within these two species. The intraspecies difference between *S. juvencus californicus* and *S. juvencus juvencus* base substitutions was 2.9%, significantly greater than some interspecific differences within genera of the Siricidae. Similarly, the intraspecific difference in CO1 gene base-substitutions between *X. spectrum spectrum* and *X. spectrum townesi* was 2.3%. Lower interspecific differences were found between *S. cyaneus* and *S. edwardsii*, *U. albicornis* and *U. californicus* and *U. cressoni* and *U. taxodii*.

The barcode sequences of other genera in the family Siricidae were less closely homologous to *S. noctilio* than other *Sirex* species. The number of base substitutions relative to *S. noctilio* increased in order of homological difference ranging from 106-123 base

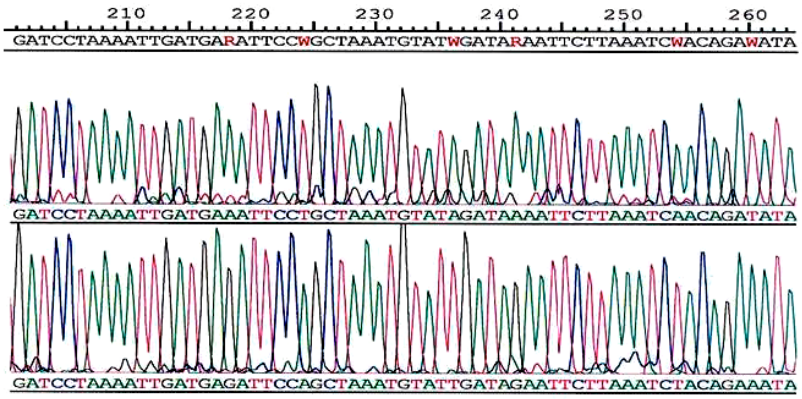

Fig. 3: Electropherogram of 60 basepair representative segment (~10%) of 650-700 basepair CO1-gene sequence showing nucleobase-substitution differences between two morphologically-similar native and exotic (non-native) *Sirex* species. Base-substitution differences in this segment are indicated here between exotic siricid woodwasp pest *Sirex noctilio* (top) compared with native siricid *Sirex cyaneus* (bottom) at positions 218, 224, 236, 241, 254 and 260 (indicated by red letters at top) in the CO1 sequence

differences or 16-18% in *Urocerus* species, to 123-138 base differences or 18-20% in *Xeris* species and 119-137 base differences or 19-20% in the tremecine species *Tremex columba* and *Eriotremex formosanus*, respectively.

Interfamilial differences in barcode sequence homology, relative to *S. noctilio*, ranged from 5-18% between members of the Siricidae and the other three families (Xiphydriidae, Orussidae and Anaxyelidae). However, barcode sequence homology differences (relative to *S. noctilio*) between these three families, outside of the Siricidae, were in a very narrow range of only 2-4% difference. Of course, this narrow range of differences, based on comparisons of barcode homology with *S. noctilio*, does not necessarily indicate the actual range of homological differences between these families if they were compared directly based on their respective CO1 gene sequences rather than their relative homologies to *S. noctilio*.

Differences in barcode sequence homology, relative to *S. noctilio*, between woodwasp species from the other three woodwasp families (Xiphydriidae, Orussidae and Anaxyelidae) were greater than differences within the Siricidae. For example, greater differences in CO1 sequence homology, relative to *S. noctilio*, were found among xiphydriid woodwasps than were found among siricid woodwasps. *Sirex noctilio* differed from two other North American xiphydriid species, *X. mellipes* and *X. tibialis*, by base substitutions of 147 of 560 positions. The single representative anaxyelid woodwasp *Syntexis libocedrii* (the cedar woodwasp) had a CO1 base substitution difference from *S. noctilio* of 173 bases or 25%, similar to the range of differences found among xiphydriid woodwasps. Adults of *S. libocedrii* are quite similar in size and shape to xiphydriid woodwasps, but have Western coniferous hosts unlike the eastern hardwood hosts of xiphydriids and consequently different mycosymbionts. The three orussid woodwasps (*O. occidentales*, *O. terminalis* and *O. thoracicus*), representatives of the Orussidae, had CO1 homology differences relative to *S. noctilio* that ranged from 161-169 bases or 23-26%.

Intrafamilial differences in sequence homology relative to *S. noctilio* within the Siricoidea were widest in the Siricidae (range 7 to 23%) among 14 species in three genera

(Table 2). The intrafamilial differences in sequence homology relative to *S. noctilio* were much narrower within the Xiphydriidae, Orussidae and Anaxyelidae families. However, interfamilial differences in sequence homology compared to *S. noctilio* were greatest between the Siricidae and the other three families. The range of interfamilial differences in percent homology (relative to *S. noctilio*) between the Xiphydriidae, Orussidae and Anaxyelidae were much lower, although these latter three families exhibited the highest barcode sequence differences from *S. noctilio*.

DISCUSSION

Sirex noctilio is considered a major invasive non-indigenous pest of pine plantations where it has been introduced in New Zealand (Nuttall, 1989), Australia (Haugen and Underdown, 1990), South America (Iede *et al*., 1998) and South Africa (Tribe and Cillie, 2004), causing high levels of economic losses until the nematode biocontrol agent *Deladenus siricidicola* (= *Beddingia siricidicola*) was introduced. This pest now poses a significant economic threat to the $8 billion softwood forest products industry in the southern United States. *Sirex noctilio* adults have been intercepted from several locations in North America prior to 2003, but there has been no evidence of an established population in any native pine forests until 2005 when an adult female was collected in a pine forest in Fulton in Oswego County, New York State about 17 km inland from Lake Ontario (Hoebeke *et al*., 2005). A report by Bergeron *et al*. (2008), indicating that *S. noctilio* adults were found at several locations across Lake Ontario in stands near the north shore in Ontario (Canada), caused concern that the original introduction of *S. noctilio* in New York State had resulted in subsequent spread to Canada via adult females flying across Lake Ontario. This was a distinct possibility because adult siricid woodwasps are known to be strong fliers capable of flying many kilometers (Talbot, 1977; Taylor, 1981; Madden, 1988). However, comparisons of the sequence homology of the rDNA internal transcribed spacer (ITS) region for New York and Ontario, Canada strains of *A. areolatum*, the internally-stored mycosymbiont of *S. noctilio*, suggest that these recent introductions in these two countries resulted from multiple woodwasp introductions rather than from vector (*S. noctilio* female) movement after one introduction (Wilson *et al*., 2009). These results indicate that the non-native, invasive *S. noctilio* woodwasp has been introduced several times in recent years in northeastern North America via infested wood importations mostly from Eurasian countries. Dodds *et al*. (2007) suggest some useful silvicultural options for reducing pine susceptibility to attack by *S. noctilio* in infested stands.

Recent analyses of 103 *Sirex* larvae (Hoebeke *et al*., 2005), intercepted from 1985-2000 by USDA-APHIS personnel at US ports of entry, confirm that most interceptions of *Sirex* species in wood were associated with cargo imported predominantly from 17 Eurasian countries, with the highest frequency of interceptions linked to crating and dunnage wood originating from Germany (39), Italy (24), China (9), Spain (7) and France (4). Most of these 103 *Sirex* larval interceptions occurred at ports of entry in Georgia (53), Kentucky (16), Florida (7), California (6) and South Carolina (4). Of the 103 *Sirex* larvae intercepted, only 7 specimens (6.8%) could be identified to species, all determined to be *S. noctilio* presumeably by association with reared adults coming out of the same wood specimens. All seven *S. noctilio* larval interceptions were derived from wood crates containing tile and marble imported from Spain and Italy. A key realization that resulted from this study was that most siricids intercepted hitherto at US ports of entry are predominantly larval stages that are seldom identifiable beyond genus due to morphologically indistinct characters. Observations

of insect port-of-entry interceptions consisting predominantly of larval stages also holds true for most other exotic insect pest species intercepted from wood at US ports of entry-based on personal interviews with many diagnostic entomologists employed at USDA-APHIS ports of entry. These observations have demonstrated the need for a standardized diagnostic method of identifying insect larval stages at ports of entry within imported wood (lumber, furniture, etc.) and in wood used as crates and dunnage for imported goods.

The present investigation of CO1 gene sequence homology between twenty-two native North American woodwasp species and invasive exotic *S. noctilio* has revealed significant differences in base substitutions within the DNA barcode to distinguish species within the four siricoid families examined. The percentage differences in base substitutions of native species relative to *S. noctilio* differed within each family and provided an indication of relatedness of taxa based on DNA sequence homology. The order of relatedness of species among woodwasp families to *S. noctilio*, based on base substitutions, followed the order of (from highest to lowest): Siricidae (Siricinae > Tremecinae) > Xiphydriidae > Orussidae > Anaxyelidae. Comparisons of CO1 gene sequences between species in the Siricidae indicated relatively high levels of homology within this family. The two native woodwasps, *Sirex cyaneus* and *Sirex edwardsii* and nonnative *Sirex juvencus juvencus* that share a close resemblance to *S. noctilio* had the highest levels of CO1-sequence homology (> 90%) with *S. noctilio*, indicated by <10% differences in base substitutions with this species. The relatedness of *S. noctilio*, compared with other genera in the Siricidae based on the CO1 gene sequence homology, followed the order of (from highest to lowest): *Sirex* > *Urocerus* > tremecines > *Xeris* species. This order of relatedness of other genera in the Siricidae to *S. noctilio* (80-90%) generally was expected given that the European woodwasp is a *Sirex* species and the morphological differences in features between adults of the other genera generally follow this same order as well. The lower levels of sequence homology (74-78%) between *S. noctilio* and the other three more distantly-related woodwasp families (Xiphydriidae, Orussidae and Anaxyelidae) also was expected, but we expected greater ranges of differences between these families and the Siricidae. The nonnative tremecine woodwasps, *Eriotremex formosanus* and *Tremex columba*, from hardwood hosts had only about 80-81% sequence homology with *S. noctilio.*

All previous molecular research done in association with siricoid woodwasps has been limited to studies of their fungal symbionts (Vasiliauskas *et al.*, 1999; Tabata *et al.*, 2000; Slippers *et al.*, 2001-2003). To our knowledge, no studies have directly compared the DNA sequence homology of diverse siricoid woodwasp species using molecular techniques, largely because of the difficulty in obtaining larval and adult specimens of this group. Furthermore, larval woodwasp specimens currently cannot be identified by morphological characters and no previous means of identifying them using any other DNA methods have been developed. Thus, complete descriptions of a woodwasp-specific modified barcode method for identifying larval specimens of siricoid woodwasps, based on sequencing of the CO1-gene, are reported here for the first time. The present study provides good evidence that sequence differences in the CO1 gene are ideal for discriminating siricoid woodwasp larvae due to significant numbers of base-substitutions that occur between species.

The relatively large (2.3 to 2.8%) CO1 gene base-substitution differences relative to *S. noctilio* found between subspecies of *Xeris spectrum* and subspecies of *Sirex juvencus* were much greater than expected for intraspecific differences. Some of these differences may be attributed to variations in the number of bases used in determining the percent difference and relative sample sizes in determining mean percent difference in base substitutions. However, base-substitution differences in the 2-3% range generally are in the range expected

between species within a family. These fairly large differences between subspecies of *Sirex juvencus* and *Xeris spectrum* indicate that taxonomic revisions probably are in order to separate these subspecies in each case into separate species. Further DNA sequence analyses will be required to determine the extent of differences between these two pairs of subspecies. *Sirex juvencus juvencus* is a European species that differs considerably in hosts, habitat and distribution from *S. juvencus californicus* which is strictly limited to Western North America.

Woodwasp immatures can live within wood for up to three years making them ideal stowaways in woody packing material from foreign countries. The capability of identifying woodwasp larvae in wood is essential for the detection of invasive exotic insect pests imported within wood products into the United States and Canada and escapes discovered in individual trees within native pine forests during pest surveys. As we increase our regulatory efforts for *S. noctilio*, we will intercept immatures in wood. Unfortunately, there are no identification keys to immatures for native or exotic siricid species (Schiff *et al*., 2006). Keys are lacking because there is very little larval material correlated with identifiable adults available for study. Since rearing adults from larval material can take up to three years, it is impractical to wait for adults to emerge before insect diagnostics are done because of the need for implementation of expedient control measures when alien species are discovered (Schiff and Wilson, 2007a, b). We have demonstrated the DNA barcode method can be effectively used to identify and discriminate siricid larvae intercepted in imported wood and larvae found in wood of standing trees during forest insect pest surveys. The DNA barcode was used to identify unique CO1-gene sequences for twenty-three native and exotic woodwasp species. None of the native woodwasp species tested had a barcode sequence identical to *S. noctilio* or to each other. DNA barcode sequences were easily obtained from fresh, frozen and preserved specimens of Siricoidea larvae and adults. All individuals within each species, collected from widely dispersed geographical regions, shared the same sequence. Sequence differences between species and genera were sufficient to allow unambiguous identification and differentiation of all woodwasp species. These sequence data will enable us to unambiguously identify a larva to species allowing pest managers to determine if an interception is of concern and the extent of an infestation. It is important to be able to conclusively identify a pest and whether it is non-native, so that decisions to apply control measures (release biocontrol agents, establish quarantines, embargoes, etc.) can be made with confidence. It is also necessary to know what effects the control measure will have on North American flora and fauna other than control of the target pest.

Several practical and theoretical problems associated with DNA barcode technologies have prompted criticism from opponents that suggest these methods are not suitable for every possible molecular diagnostic application. Use of this short stretch of mitochondrial DNA sequence as a universal identifier for animal taxa (including insects) has met with considerable opposition from some scientists (Lipscomb *et al*., 2003; Sperling, 2003; Will and Rubinoff, 2004; Meyer and Paulay, 2005; DeSalle *et al*., 2005; Will *et al*., 2005). Several possible theoretical problems associated with DNA barcode use have been proposed. It has been suggested that mtDNA sequences are not necessarily representative in providing a precise reflection of species boundaries due to intraspecific geographical variation in nuclear sequences, morphology, mating preferences, host specificity and other biological attributes (Brower, 2006). Furthermore, there is the possibility of overlap between intraspecific and interspecific variation (Crochet *et al*. 2003; Penton *et al*., 2004; Meyer and Paulay, 2005). Thus, sequence variation does not necessarily indicate biological differences or correspond to existing Linnean binomials (Ebach and Holdrege, 2005). Sometimes only single-fragment

mtDNA are available or used in analyses that limit the thoroughness of sequence comparisons (Lipscomb *et al*., 2003; Mallet and Willmott, 2003; Will and Rubinoff, 2004). Unrecognized cryptic species may confound species recognition (Funk and Omland, 2003; Meyer and Paulay, 2005; Monaghan *et al*., 2006), especially when the criteria used for species delimitation are applied inconsistently (Cracraft, 1992; Wiens and Penkrot, 2002; Agapow *et al*., 2004). Finally, BLAST searches of NCBI GenBank databases only associate the unknown sequence with its closes known matches which are limited by the representativeness or adequacy of sampling, accuracy of sequences deposited and associated annotations and uniformity of the process of translating sequence comparisons into species names (Moritz and Cicero, 2004; Meyer and Paulay, 2005; Nilsson *et al*., 2006).

Many more proponents of DNA barcode applications appear to be surfacing than opponents in recent years. This movement in favor of using the DNA barcode appears to be resulting from the need to develop universal, standardized molecular methods for building worldwide-accessible diagnostic databases and to build directly-comparable data of a rapidly increasing number of diverse species in order to more precisely define taxonomic and phylogenetic relationships between taxa. Obviously, this is only possible when the molecule of choice being sequenced is universally the same. As a result, the DNA barcode method has been widely accepted and is now being used by major museums and funding agencies for wholesale identifications (Brower, 2006).

Numerous advantages of utilizing DNA barcodes for species identifications have been recognized in the area of biosecurity, particularly for the identification of Invasive Alien Species (IAS) such as the *Sirex noctilio* woodwasp. The use of internationally-standardized technologies for identifying IAS have been recommended in order to create a framework for more effective, coordinated and coherent efforts within the global IAS diagnostic community (Armstrong and Ball, 2005). The current molecular diagnostic tools available for IAS identification are very limited and not flexible enough to accommodate the very diverse taxonomic range of invasive exotic pest species even though molecular methods are considered essential components of the biosecurity toolbox (Martin *et al*., 2000). Unlike such PCR-based technologies as Restriction Fragment-Length Polymorphism (RFLP), multiplex PCR and oligonucleotide array analysis, DNA barcoding is less limited because it uses a single-gene region common across all taxa with no inconsistency in the gene or parts of the genes used for species identification. Perhaps the most important advantage of the barcode method over all other diagnostic methods is the ability to continually add in more species (Armstrong and Ball, 2005). Thus, idiosyncrasies of different methods are avoided and DNA barcoding methods can be standardization across species and laboratories allowing for a global exchange of homologous data. Other noted advantages of DNA barcoding include the ability to compare against unlimited reference sequences, to utilize all of the genetic data (not just small snippets of data at priming or restriction sites) and to build an appropriate reference dataset over a relatively short time period. Furthermore, the tree-based approach enables all of the data to be observed simultaneously. Employing adult reference specimens facilitates the identification of morphologically-indistinct immature larval stages to complement the morphological approach to insect diagnoses. A global-scale effort to obtain validated reference specimens permits the building of robust reference profiles for quality control of sequence data, increasing confidence in data sharing and analyses and allowing standardized criteria for interpreting barcode results (Armstrong and Ball, 2005). This approach provides the potential for a globally collaborative, unified and less fragmented approach to molecular diagnostics for international biosecurity, allowing for a level of transparency for species identifications across countries that is not currently possible using

different diagnostic methods. With DNA barcoding, the absence of appropriate reference specimens for identifying unknowns leads to non-identifications, not misidentifications. Consequently, false positives are avoided and the existence of new or cryptic species are identified.

The benefits of capitalizing on the efforts of many scientists worldwide using standardized molecular technologies do not eliminate the occurrence of situations where DNA barcoding is not sufficient to solve certain identification problems. Thus, certain other molecular tools such as species-specific primer tests for insect species identifications would not be replaced by DNA barcoding (Stewart *et al*., 2009). However, DNA barcoding offers many advantages over most other available molecular technologies for the development of a internationally-standardized global collaborative approach to systematic molecular diagnostics.

A major reason for identifying an organism is to connect it to existing knowledge about the group of which it is a member and integrate new observations into that general context (Brower, 2006). The definition of populations based on individual organisms rather than a preconceived interbreeding group generally is more objective for species delimitations (Vrana and Wheeler, 1992). To facilitate the cataloguing of very large numbers of undescribed insect species, Pons *et al*. (2006) proposed the use of a method that determines the locations of ancestral nodes in order to define putative species for large-scale species discovery from sequence data. An alternative approach for detecting species separations is to search for discontinuities in sequence variation associated with species boundaries such as by using statistical parsimony analysis (Templeton *et al*., 1992). Parsimony analysis, commonly used on DNA barcode data, separates groups of sequences into different sequence networks when genotypes are connected by comparatively long branches that are affected by homoplasy. This algorithm in practice is often effective in separating groups that are roughly coincidental with named species or species groups even though homoplasious connections do not necessarily correspond to species boundaries (Templeton, 2001; Wilder and Hollocher, 2003; Cardoso and Vogler, 2005).

DNA barcoding provides perhaps its greatest utility in distinguishing morphologically identical taxa and the early developmental stages of insects that often possess very few if any morphologically-identifiable characters to distinguish species. The present work has demonstrated DNA barcode methods can be used to identify larval states of woodwasps in wood as easily as free-flying adults. With DNA barcoding methods, we are now able to identify larvae by sequence comparisons. Through conclusive identifications of intercepted larval specimens, this molecular diagnostic tool should help prevent future introductions of *S. noctilio* and other exotic woodwasps, especially in the more susceptible pines of the Southern United States and possibility many other potentially susceptible western pine species.

ACKNOWLEDGMENTS

This research was funded by a 3-year Special Technology Development Program (STDP) grant R8-2005 provided by the USDA Forest Service, Forest Health Protection (FHP), received through the Southern Research Station (SRS). We thank FHP personnel Don Duerr and Noel Schneeburger for assistance with funding; David Smith, Kevin Dodds, Edson Tadeu Iede, Dick Bashford and Vicky Klasmer for woodwasp adults, larvae and woodwasp-infested wood collections; and Dennis Haugen for photographs and advice. We give special

thanks to Brian Scheffler (USDA-ARS), Amy Abel and Charisse Oberle for processing DNA samples and Doug Streett and Meg Allen for use of the USDA-ARS Bldg. 8 quarantine facility in Stoneville, MS.

REFERENCES

Agapow, P.M., O.R.P. Bininda-Emonds, K.A. Crandall, J.L. Gittleman, G.M. Mace, J.C. Marshall and A. Purvis, 2004. The impact of species concept on biodiversity studies. Quart. Rev. Biol., 79: 161-179.

Armstrong, K.F., C.M. Cameron and E.R. Frampton, 1997. Fruit fly (Diptera: Tephritidae) species identification: A rapid molecular diagnostic technique for quarantine application. Bull. Entomol. Res., 87: 111-118.

Armstrong, K.F., P. McHugh, W. Chinn, E.R. Frampton and P.J. Walsh, 2003. Tussock moth species arriving on imported used vehicles determined by DNA analysis. N. Z. Plant Prot., 56: 16-20.

Armstrong, K.F. and S.L. Ball, 2005. DNA barcodes for biosecurity: Invasive species identification. Philos. Trans. R. Soc. B, 360: 1813-1823.

Bergeron, J.J., R.C. Hamelin, I. Leal, C. Davis and P. de Groot, 2008. First report of *Amylostereum areolatum*, the fungal symbiont of *Sirex noctilio*, on *Pinus* spp. in Canada. Plant Dis., 92: 1138-1138.

Brower, A.V.Z., 2006. Problems with DNA barcodes for species delimitation: Ten species of *Astraptes fulgerator* reassessed (Lepidoptera: Hesperiidae). Syst. Biodiversity, 4: 127-132.

Cardoso, A. and A.P. Vogler, 2005. DNA taxonomy, phylogeny and Pleistocene diversification of the *Cicindela hybrida* species group (Coleoptera: Cicindelidae). Mol. Ecol., 14: 3531-3546.

Cock, M.J.W., M. Kenis and R. Wittenburg, 2003. Biosecurity and forests: An introduction with particular emphasis on forest pests. FAO Forest Health and Biosecurity Working Paper FBS/2E. Forest Resources Development Service, pp: 2-4. http://www.fao.org/docrep/006/j1467e/j1467e00.htm.

Cracraft, J., 1992. The species of the birds-of-paradise (Paradisaeidae): Applying the phylogenetic species concept to complex pattern of diversification. Cladistics, 8: 1-43.

Crochet, P.A., J.Z. Chen, J.M. Pons, J.D. Lebreton, P.D.N. Hebert and F. Bonhomme, 2003. Genetic differentiation at nuclear and mitochondrial loci among large white-headed gulls: Sex-biased interspecific gene flow. Evolution, 57: 2865-2878.

DeSalle, R., M.G. Egan and M. Siddall, 2005. The unholy trinity: Taxonomy, species delimitation and DNA barcoding. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci., 360: 1905-1916.

Dodds, K.J., R.R. Cooke and D.W. Gilmore, 2007. Silvicultural options to reduce pine susceptibility to attach by a newly detected invasive species, *Sirex noctilio*. North J. Appl. For., 24: 165-167.

Dugdale, J.S., D. Gleeson, L.H. Clunie and P.W. Holder, 2005. A Diagnostic Guide to Tortricidae Encountered in Field Surveys and Quarantine Inspections in New Zealand: Morphological and Molecular Characters. Ministry of Agriculture and Forestry, Wellington, New Zealand, pp: 163.

Ebach, M.C. and C. Holdrege, 2005. DNA barcoding is no substitute for taxonomy. Nature, 434: 697-697.

Funk, D.J. and K.C. Omland, 2003. Species-level paraphyly and polyphyly: Frequency, causes and consequences, with insights from animal mitochondrial DNA. Ann. Rev. Ecol. Syst., 34: 397-423.

Haugen, D.A. and E.R. Hoebeke, 2005. Sirex woodwasp *Sirex noctilio* F. (Hymenoptera: Siricidae). USDA Forest Service, Northeastern Area, State and Private Forestry, Newtown Square, PA, Pest Alert NA-PR-07-05.

Haugen, D.A. and M.G. Underdown, 1990. *Sirex noctilio* control program in response to the 1987 Green Triangle outbreak. Aust. For., 53: 33-40.

Hebert, P.D.N., A. Cywinska, S.L. Ball and J.R. de Waard, 2003. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B, 270: 313-321.

Hoebeke, E.R., D.A. Haugen and R.A. Haack, 2005. *Sirex noctilio*: Discovery of a palearctic siricid woodwasp in New York. Newslett. Mich. Ent. Soc., 50: 24-25.

Hurley, B.P., B. Slippers and M.J. Wingfield, 2007. A comparison of control results for the alien invasive woodwasp, *Sirex noctilio*, in the southern hemisphere. Agric. For. Ent., 9: 159-171.

Iede, E.T., S.R.C. Penteado and E.G. Schaitza, 1998. *Sirex noctilio* problem in Brazil: detection evaluation and control. Proceeding of the a Conference Training in the Control of *Sirex noctilio* by the Use of Natural Enemies, (CTCNE'98), USDA Forest Service, FHTET 98-13, pp: 45-52.

Lipscomb, D., N. Platnick and Q. Wheeler, 2003. The intellectual content of taxonomy: A comment on DNA taxonomy. Trends Ecol. Evol., 18: 65-66.

Mack, R.N., D. Simberloff, W.M. Lonsdale, H. Evans, M. Clout and F.A Bazzaz, 2000. Biotic invasions: Causes, epidemiology, global consequences and control. Ecol. Appli., 10: 689-710.

Madden, J.L., 1988. *Sirex* in Australasia. In: Dynamics of Forest Insect Populations, Berryman, A.A. (Ed.). Plenum, New York, pp: 407-429.

Mallet, J. and K. Willmott, 2003. Taxonomy: Renaissance or tower of babel? Trends Ecol. Evol., 18: 57-59.

Martin, R.R., D. James and C.A. Lévesque, 2000. Impacts of molecular diagnostic technologies on plant disease management. Ann. Rev. Phytopathol., 38: 207-239.

McCullough, D.G., T.T. Work, J.F. Cavey, A.M. Liebhold and D. Marshall, 2006. Interceptions of nonindigenous plant pests at US ports of entry and border crossings over a 17-year period. Biol. Invasions, 8: 611-630.

Meyer, C.P. and G. Paulay, 2005. DNA barcoding: Error rates based on comprehensive sampling. PLoS Biol., 3: 2229-2238.

Monaghan, M.T., M. Balke, J. Pons and A.P. Vogler, 2006. Beyond barcodes: Complex DNA taxonomy of a South Pacific Island radiation. Proc. R. Soc. B, 273: 887-893.

Moritz, C. and C. Cicero, 2004. DNA barcoding: Promise and pitfalls. PLoS Biol., 2: 354-354.

Nilsson, R.H., M. Ryberg, E. Kristiansson, K. Abarenkov, K. Larsson and U. Kôljalg, 2006. Taxonomic reliability of DNA sequences in public sequence databases: A fungal perspective. PLoS One, 1: 59-59.

Nuttall, M.J., 1989. *Sirex noctilio* Sirex Wood Wasp (Hymenoptera: Siricidae). In: Review of Biological Control of Invertebrate Pests and Weeds in New Zealand from 1874 to 1986, Cameron, P.J., R.L. Hill, J. Bain and W.P. Thomas (Eds.). CAB International Institute of Biological Control, London, pp: 299-306.

Penton, E.H., P.D.N. Hebert and T.J. Crease, 2004. Mitochondrial DNA variation in North American populations of *Daphnia obtusa*: Continentalism or cryptic endemism? Mol. Ecol., 13: 97-107.

Pimentel, D., L. Lach, R. Zuniga and D. Morrison, 2000. Environmental and economic costs of nonindigenous species in the United States. Bioscience, 50: 53-65.

Pons, J., T.G. Barraclough, J. Gomez-Zurita, A. Cardoso and D.P. Duran *et al.*, 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. Syst. Biol., 55: 595-595.

Schiff, N.M., S.A. Valley, J.L. Bonte and D.R. Smith, 2006. Guide to the Siricid Woodwasps of North America. USDA Forest Health Technology Enterprise Team, Morgantown, West Virginia, USA., pp: 102.

Schiff, N.M. and A.D. Wilson, 2007a. Controlling *Sirex noctilio* in North America a new paradigm. Proceedings of the 17th U.S. Department of Agriculture Interagency Research forum on Gypsy Moth and other Invasive Species 2006, Jan. 10-13, U.S. Department of Agriculture, Forest Service, Northern Research Station, pp: 87-87.

Schiff, N.M. and A.D. Wilson, 2007b. Siricid diversity, biology and tools for identification. Proceedings of International *Sirex* Symposium And Workshop, (ISW'07), Pretoria, South Africa, pp: 64-64.

Slippers, B., M.J. Wingfield, B.D. Wingfield and T.A. Coutinho, 2001. Population structure and possible origin of *Amylostereum areolatum* in South Africa. Plant Pathol., 50: 206-210.

Slippers, B., B.D. Wingfield, T.A. Coutinho and M.J. Wingfield, 2002. DNA sequence and RFLP data reflect relationships between *Amylostereum* species and their associated wood wasp vectors. Mol. Ecol., 11: 1845-1854.

Slippers, B., T.A. Coutinho, B.D. Wingfield and M.J. Wingfield, 2003. The genus *Amylostereum* and its association with wood wasps: A contemporary review. South Afric. J. Sci., 99: 70-74.

Smith, M.A., D.M. Wood, D.H. Janzen, W. Hallwachs and P.D.N. Hebert, 2007. DNA barcodes affirm that 16 species of apparently generalist tropical parasitoid flies (Diptera, Tachinidae) are not all generalists. Proc. Natl. Acad. Sci. USA., 104: 4967-4972.

Sperling, F.A.H., 2003. DNA barcoding: Deus ex machina. Newslett. Biol. Survey Canada, 22: 50-53.

Stewart, L.C., R.J. Hale and M.L. Hale, 2009. Species-specific primers for the molecular identification of cryptic *Bombus* species in New Zealand. Conserv. Genet., 10: 1-3.

Tabata, M., T.C. Harrington, W. Chen and Y. Abe, 2000. Molecular phylogeny of species in the genera *Amylostereum* and *Echinodontium*. Mycoscience, 41: 585-593.

Talbot, P.H.B., 1977. The *Sirex-amylostereum-pinus* association. Ann. Rev. Phytopathol., 15: 41-54.

Taylor, K.L., 1981. The *Sirex* Woodwasp: Ecology and Control of an Introduced Forest Insect. In: The Ecology of Pests-Some Australian Case Histories, Kitching, R.L. and R.E. Jones (Eds.). CSIRO, Melbourne.

Templeton, A.R., K.A. Crandall and C.F. Sing, 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and sequencing data. III. Cladogram estimation. Genetics, 132: 619-633.

Templeton, A.R., 2001. Using phylogeographic analyses of gene trees to test species status and processes. Mol. Ecol., 10: 779-791.

Toda, S. and S. Komazaki, 2002. Identification of thrips species (Thysanoptera: Thripidae) on Japanese fruit trees by polymerase chain reaction and restriction fragment length polymorphism of the ribosomal ITS2 region. Bull. Entomol. Res., 92: 359-363.

Tribe, G.D. and J.J. Cillie, 2004. The spread of *Sirex noctilio* Fabricius (Hymenoptera: Siricidae) in South African pine plantations and the introduction and establishment of its biological control agents. Afr. Entomol., 12: 9-17.

Vasiliauskas, R., H. Johannesson and J. Stenlid, 1999. Molecular relationships within the genus *Amylostereum* as determined by ITS sequences of the ribosomal DNA. Mycotaxon, 71: 155-161.

Vrana, P. and W.C. Wheeler, 1992. Individual organisms as terminal entities: Laying the species problem to rest. Cladistics, 8: 67-72.

Wiens, J.J. and T.A. Penkrot, 2002. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (Sceloporus). Syst. Biol., 51: 69-91.

Wilder, J.A. and H. Hollocher, 2003. Recent radiation of endemic Caribbean Drosophila of the dunni subgroup inferred from multilocus DNA sequence variation. Evolution, 57: 2566-2579.

Will, K.W. and D. Rubinoff, 2004. Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. Cladistics, 20: 47-55.

Will, K.W., B.D. Mishler and Q.D. Wheeler, 2005. The perils of DNA barcoding and the need for integrative taxonomy. Syst. Biol., 54: 844-851.

Williamson, M., 1996. Biological Invasions. Chapman Hall, London, pp: 244.

Wilson, A.D. and N.M. Schiff, 2000a. Somatic antagonism between fungal symbionts of xiphydriid woodwasps. Phytopathology, 90: 84-84.

Wilson, A.D. and N.M. Schiff, 2000b. Xylariaceous wood decay fungi: Mycosymbionts of xiphydriid woodwasps. Phytopathology, 90: 84-84.

Wilson, A.D. and N.M. Schiff, 2003. Wood decay potential of mycosymbionts from xiphydriid and tremecine woodwasps. Phytopathology, 93: 90-90.

Wilson, A.D., T.D. Leininger, W.J. Otrosina, L.D. Dwinell and N.M. Schiff, 2004. The Impact and Control of Major Southern Forest Diseases. In: Southern Forest Science: Past, Present and Future, Rauscher, H.M. and K. Johnsen (Eds.). Chapter 16, U.S. Department of Agriculture, Forest Service, Southern Research Station, Asheville, North Carolina, pp: 161-178.

Wilson, A.D. and N.M. Schiff, 2006. Molecular methods for the detection of *Sirex noctilio* woodwasp larvae, vectors of *Amylostereum areolatum* in North American pines. Phytopathology, 96: S123.

Wilson, A.D., N.M. Schiff, D.A. Haugen and E.R. Hoebeke, 2009. First report of *Amylostereum areolatum* in pines in the United States. Plant Dis., 93: 108-109.

Yemshanov, D., D.W. McKenney, P. de Groot, D. Haugen, D. Sidders and B. Joss, 2008. An assessment of *Sirex noctilio* spread and potential impacts on pine wood supply and harvests in eastern Canada. Proceedings of the 19th U.S. Department of Agriculture Interagency Research Forum on Invasive Species 2008, (DAIRFIS'08), USDA Forest Service, Newtown Square, PA. GTR-NRS-P-36. pp: 88-90.

Yemshanov, D., D.W. McKenney, P. de Groot, D. Haugen, D. Sidders and B. Joss, 2009. A bioeconomic approach to assess the impact of an alien invasive insect on timber supply and harvesting: A case study with *Sirex noctilio* in eastern Canada. Can. J. For. Res., 39: 154-168.