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# DNA barcodes and molecular diagnostics to distinguish an introduced and native *Laricobius* (Coleoptera: Derodontidae) species in eastern North America

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ABSTRACT

Molecular diagnostics based on DNA barcodes can be powerful identification tools in the absence of distinctive morphological characters for distinguishing between closely related species. A specific example is distinguishing the endemic species *Laricobius rubidus* from *Laricobius nigrinus*, a biological control agent of hemlock woolly adelgid introduced into the eastern United States. This is especially important because their larvae are morphologically similar and are often collected together in stands where pines and hemlock grow together. Diagnostic nucleotide differences to distinguish species were determined using 157 *L. nigrinus* and 205 *L. rubidus* cytochrome oxidase I (COI) barcode DNA sequences. Two polymerase chain reaction (PCR) assays were developed: PCR followed by restriction length polymorphism (RFLP) and real-time PCR (qPCR) based on hydrolysis probes. The qPCR assay had limited success when applied to *L. nigrinus* originating from inland vs. coastal populations in the western United States. PCR–RFLP was successful, regardless of sample origin. These two species-specific assays provide a choice of diagnostic tools to best match the available lab equipment and management objectives for those using *L. nigrinus* as a biological control agent.



## 1. Introduction

Molecular diagnostics are increasingly recognized as powerful tools to facilitate biological control programs (Gariepy et al., 2007). They can provide accurate and relatively inexpensive identification of natural enemies to track their establishment, deduce population structure, and monitor impacts on non-target organisms. Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) has been used only recently as a tool in biological control programs. Examples include distinguishing native from introduced *Aphelinus* parasitoid species in South Africa (Prinsloo et al., 2002), and discrimination of cryptic *Encarsia* species used for biological control of whiteflies (Monti et al., 2005). Likewise, species discrimination of juvenile insects via real-time PCR (qPCR) has been applied to economically important herbivores (e.g., Kox et al., 2005; Feng et al., 2007), and for *Ceratapion* species in biological control of yellow starthistle (Antonini et al., 2009). Assays based on qPCR have also been used to identify and quantify consumption of prey species by predators (Zhang et al., 2007; Weber and Lundgren, 2009). Hebert et al. (2003) suggested the use of a 650 bp region within the mitochondrial cytochrome oxidase subunit I (COI) gene as a standard for species identification and referred to it as a DNA barcode. Benefits of using the COI gene are that universal primers can be used for most animals and it is variable enough to distinguish many taxa (Hebert et al., 2004a). Variation within the COI gene of different geographic populations within species is common and can be addressed by including samples from each population in a DNA barcode library (Moritz and Cicero, 2004).

*Laricobius nigrinus* Fender is endemic to western North America where it is a predator of hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae) (Lawrence and Hlavac, 1979; Zilahi-Balogh et al., 2002; Mausel, 2005; Kohler et al., 2008). *A. tsugae* is native to Asia and western North America on seven of the nine existing *Tsuga* species, where it is not considered a major pest (Annand, 1924; Farjon, 1990; Cheah et al., 2004; Havill et al., 2006). In eastern North America, *Tsuga canadensis* (L.) Carrière and *Tsuga carolinia* Engelm. are threatened by an invasion of *A. tsugae* from Japan (Havill et al., 2006). *L. nigrinus* was introduced into the eastern United States in 2003 as part of a biological control program against *A. tsugae* (Lamb et al., 2006). Over 100,000 *L. nigrinus* adults have been released at over 120 sites in 14 eastern states (Roberts et al., 2011, unpublished data).

Surveys of natural enemies associated with *A. tsugae* in eastern North America showed that existing predators were not capable of reducing pest populations below lethal levels (Montgomery and Lyon, 1996; Wallace and Hain, 2000). One native predator, *Laricobius rubidus* LeConte, associated with pine bark adelgid,

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*Pineus strobi* (Hartig), on eastern white pine, *Pinus strobus* L. (Clark and Brown, 1960), is also capable of completing its lifecycle on *A. tsugae*, yet prefers pine bark adelgid to *A. tsugae* for oviposition (Zilahi-Balogh et al., 2005). It is common to recover both *L. nigrinus* and *L. rubidus* during sampling for the presence of *L. nigrinus* on *A. tsugae*-infested hemlock trees (Mausel et al., 2010).

Mausel et al. (2010) found sampling for immature life stages of *L. nigrinus* was more informative than adult sampling. *Laricobius* larvae are morphologically indistinguishable and therefore must be reared to adults for identification. However, rearing the beetles in the laboratory is labor intensive with a high rate of mortality during the pupal stage (Lamb et al., 2005). This paper describes DNA barcode development and two PCR-based assays for discriminating *L. nigrinus* from *L. rubidus* in all life stages. Assays were also performed using *A. tsugae* and *P. strobi* to determine whether signals generated from these prey species in the guts of *Laricobius* samples would confound identification.

## 2. Materials and methods

### 2.1. Beetle collection and DNA extraction

157 *L. nigrinus* specimens were collected from western North America (Idaho, Washington, Oregon, Montana, and British Columbia), and 205 *L. rubidus* samples were collected from eastern USA (Connecticut, Virginia, Pennsylvania, Minnesota, West Virginia, Tennessee, Massachusetts, North Carolina, Maryland, Kentucky, and Georgia) from 2006 to 2008 (Table 1). They were preserved in 95–100% ethanol then stored at −20 °C until DNA extraction. Beetle abdomens were removed for genomic DNA extraction using the DNA IQ extraction kit (Promega, Madison, WI). Beetle heads, thoraces, and male genitalia were saved as vouchers and deposited at Yale University's Peabody Museum of Natural History.

### 2.2. DNA barcoding

Partial cytochrome oxidase subunit I (COI) was amplified for all specimens using forward primer LepF1 and reverse primer LepR1 (Hebert et al., 2004b). PCR was performed in 30 μl reactions containing 3.0 μl 10× PCR Buffer, 2.4 μl dNTPs (10 mM), 3.0 μl $MgCl_2$ (25 mM), 1.0 μl BSA (10 mg/ml), 1.0 μl of each primer (10 mM), 0.3 μl Taq DNA polymerase (Qiagen Inc., Valencia, CA), and 1.0 μl of DNA template. Thermocycling conditions were 95 °C for 5 min followed by 35 cycles of 45 s at 95 °C, 45 s at 48 °C, and 1 min at 72 °C, with a final extension of 72 °C for 5 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). Sequencing reactions were performed using the BigDye Terminator kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied Biosystems 3730 automated sequencer. Sequences were edited and aligned using Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, MI). All sequences were deposited in GenBank (Table 1). Sequence divergences were calculated using the Kimura 2-parameter model (Kimura, 1980) using PAUP* (Swofford, 2003). Relationships among COI haplotypes were examined by constructing a network based on the statistical parsimony method of Templeton et al. (1992) using the software TCS 1.21 (Clement et al., 2000).

### 2.3. PCR–RFLP

A subset of the 362 beetles that were sequenced for COI was assayed using PCR–RFLP as an example of specimens potentially collected during *L. nigrinus* post-release monitoring. *L. nigrinus* samples were chosen from populations that were used as the source of introductions to the eastern United States (HM803547–HM803557, HM8035530–HM803554, HM803572–HM803577, HM8035880–HM803586), and *L. rubidus* samples were from within the *A. tsugae*-infested range (HM803591, HM803594, HM803596–HM803598, HM803600, HM803613–HM803615, HM803619, HM803621–HM803624, HM803627, HM803632–HM803635, and JF700523. DNA from 20 individuals of each species ($n = 40$) was amplified using the PCR conditions as described above. Twelve and a half microliters of PCR product were added to each of two RFLP reactions containing either 0.5 μl AseI and 1.5 μl 10× Buffer 3, or 0.5 μl HpaII and 1.5 μl 10× Buffer 1 (New England BioLabs, Ipswich, MA). Incubation at 37 °C for 2 h was followed by gel electrophoresis and visualization on a 2% agarose gel.

### 2.4. qPCR

Real-time PCR was performed on the same 40 samples as above. A custom TaqMan single nucleotide polymorphism (SNP) genotyping assay was designed using Primer Express software v2.0 (Applied Biosystems, Foster City, CA). The assay contained AmpliTaq Gold DNA polymerase, ROX labeled passive reference dye, primers LnF (5′ CATAGTAATACCTATTATAATTGGTGGATTTGG 3′) and LnR (5′ GGAGGTAGTATTCAGAATCTTATATTATTTATTCG 3′), and minor binding groove (MBG) hydrolysis probes VIC (5′ TAATACTAGGGG CTCCTGAT 3′) specific to *L. nigrinus* and FAM (5′ TAATACTAG GAGCTCCCGAT 3′) specific to *L. rubidus*. Primers correspond to the 157–189 and 239–273 positions of the archived barcode samples. Probes correspond to positions 207–226 and were designed with two diagnostic nucleotides at positions 217 and 223. Template DNA was quantified with a NanoDrop 1000 spectrometer (Thermo Fisher Scientific, Wilmington, DE) and aliquots were diluted to 10 ng/μl before amplification. To account for possible pipetting error, duplicate amplification reactions contained 10.0 μl TaqMan genotyping master mix (Applied Biosystems, Foster City, CA), 1.0 μl (20×) custom TaqMan SNP genotyping assay, 1.0 μl genomic DNA template, and 8 μl water, totaling 20 μl reactions. Negative controls with no template were included as a check for contamination.

Experimental protocols followed the manufacturer's recommendations for the StepOne™ Real-Time PCR instrument and software version 2.0 (Applied Biosystems, Foster City, CA). Assay assignment designated allele 1 as VIC and allele 2 as FAM. Thermocycling conditions were: pre-PCR read for 30 s at 60 °C, holding stage for 10 min at 95 °C, cycling stage of 15 s at 95 °C followed by 1 min at 60 °C for a total of 40 cycles, and post PCR read for 30 s at 60 °C. Change in detected fluorescence was displayed on a bivariate plot to distinguish degrees of homo- and heterozygosity. The genotyping program assignment of a homozygote was equivalent to assignment of one of the two species' haploid mtDNA template. The program default assigned one of the species as a heterozygote. Therefore, the default analysis settings were changed, turning on 2-cluster calling, and heterozygotes were manually called homozygote for allele 2.

### 2.5. Prey species

*A. tsugae* and *P. strobi* are adelgid species that are the most likely prey to be present in the guts of *L. nigrinus* and *L. rubidus* samples. Samples of *A. tsuage* were collected from eastern hemlock in Litchfield County, Connecticut, and Randolph County, West Virginia. *P. strobi* were collected from white pine in Lincoln County, Maine and Suffolk County, Massachusetts. Vouchers are deposited at the Canadian National Collection of Insects in Ottawa. Adelgids were preserved in 95% ethanol, and stored at −20 °C until DNA extraction. Total DNA was extracted from two samples for each species using the DNeasy blood and tissue kit (Qiagen Inc., Velencia, CA). DNA barcodes were produced from these samples as part of a previous study (Foottit et al., 2009). PCR–RFLP and qPCR were completed for each sample as previously described. Samples of *L. nigrinus* and *L. rubidus* served as positive controls.

**Table 1**
Specimen DNA barcode and collection information.

| Genbank Accession No. | # of specimens | Species | Collection information |
|---|---|---|---|
| HM803283–HM803287 | 6 | *L. nigrinus* | USA; WA; King County; June 2006; Coll. D. Mausel |
| HM803310–HM803317 | 8 | *L. nigrinus* | USA; OR; Multnomah County; Portland; 23 January 2006; Coll. D. Ross |
| HM803404–HM803411 | 8 | *L. nigrinus* | USA; WA; King County; Seattle; 18 January 2007; Coll. D. Mausel, C. Jubb |
| HM803412–HM803433 | 22 | *L. nigrinus* | USA; ID; Kootenai County; Coeur d'Alene; 9 March 2007; Coll. D. Mausel |
| HM803436 | 1 | *L. nigrinus* | USA; OR; Marion County; 2 May 2007; Coll. G. Kohler |
| HM803437–HM803438 | 2 | *L. nigrinus* | USA; OR; Polk County; Bethel Heights; 18 April 2007; Coll. G. Kohler |
| HM803439 | 1 | *L. nigrinus* | CANADA; British Columbia; Vernon; 10 May 2007; Coll. G. Zilahi-Balogh |
| HM803440–HM803443 | 4 | *L. nigrinus* | USA; ID; Latah County; Moscow; 9 November 2007; Coll. D. Mausel, S. Cook |
| HM803444 | 1 | *L. nigrinus* | USA; OR; Polk County; Bethel Heights; 26 October 2007; Coll. G. Kohler |
| HM803445–HM803451 | 7 | *L. nigrinus* | USA; WA; Thurston County; Olympia; 26 October 2007; Coll. G. Kohler |
| HM803452–HM803460 | 9 | *L. nigrinus* | USA; WA; King County; Seattle; January 2007; Coll. D. Mausel, C. Jubb |
| HM803468–HM803470 | 3 | *L. nigrinus* | CANADA; British Columbia; Vernon; August 2007; Coll. J.E. Corrigan |
| HM803472 | 1 | *L. nigrinus* | USA; WA; King County; Seattle; 7 April 2008; Coll. R. McDonald |
| HM803505–HM803516 | 12 | *L. nigrinus* | USA; WA; Pierce County; Tacoma; 12 May 2008; Coll. M.E. Montgomery, R. McDonald |
| HM803517–HM803545 | 29 | *L. nigrinus* | USA; ID; Kootenai County; Coeur d'Alene; 2–10 November 2007; Coll. D. Mausel |
| HM803546–HM803571 | 26 | *L. nigrinus* | USA; ID; Latah County; Moscow; 8–9 November 2007; Coll. D. Mausel |
| HM803572–HM803577 | 6 | *L. nigrinus* | USA; WA; King County; Seattle; November 2007; Coll. R. McDonald |
| HM803580–HM803586 | 7 | *L. nigrinus* | USA; OR; Multnomah County; Portland; 25 October 2008; Coll. D. Ross; K. Wallin |
| HM803587–HM803590 | 4 | *L. nigrinus* | USA; MT; Flathead County; West Glacier; 6 October 2008; Coll. D. Mausel |
| HM803308–HM803309 | 2 | *L. rubidus* | USA; VA; Montgomery County; 7 June 2006; Coll. D. Mausel |
| HM803318–HM803324 | 7 | *L. rubidus* | USA; WV; Pocahontas County; Seneca State Forest; 27 April 2006; Coll. D. Mausel |
| HM803325–HM803336 | 12 | *L. rubidus* | USA; WV; Pocahontas County; Watoga State Park; 27 April 2006; Coll. D. Mausel |
| HM803337–HM803339 | 3 | *L. rubidus* | USA; NC; Watauga County; Foscoe; 16 April 2006; Coll. D. Mausel |
| HM803340–HM803341 | 2 | *L. rubidus* | USA; NC; Yancey County; Locust Creek; 16 April 2006; Coll. D. Mausel |
| HM803343–HM803345, HM803347, HM803349–HM803355 | 11 | *L. rubidus* | USA; TN; Blount County; Great Smoky Mountains National Park; 9 April 2006; Coll. D. Mausel |
| HM803356–HM803358, HM803402 | 4 | *L. rubidus* | USA; MD; Allegany County; Rocky Gap State Park; 3 May 2006; Coll. D. Mausel |
| HM803359–HM803360 | 2 | *L. rubidus* | USA; PA; Union County; Bald Eagle State Forest; 4 May 2006; Coll. D. Mausel |
| HM803361, HM803363–HM803365 | 4 | *L. rubidus* | USA; PA; Huntingdon County; Rothrock State Forest; 4 May 2006; Coll. D. Mausel |
| HM803366–HM803373 | 8 | *L. rubidus* | USA; VA; Smyth County; Hurricane Camp; 20 April 2006; Coll. D. Mausel |
| HM803374 | 1 | *L. rubidus* | USA; VA; Bland County; Lick Creek; 18 April 2006; Coll. D. Mausel |
| HM803375–HM803376 | 2 | *L. rubidus* | USA; VA; Smyth County; Dickey Creek; 20 April 2006; Coll. D. Mausel |
| HM803377–HM803382 | 6 | *L. rubidus* | USA; VA; Montgomery County; Kentland Farm; 28 April 2006; Coll. D. Mausel |
| HM803383–HM803388, HM803390–HM803392 | 9 | *L. rubidus* | USA; VA; Giles County; North Fork; 15 April 2006; Coll. D. Mausel |
| HM803393 | 1 | *L. rubidus* | USA; VA; Grayson Co.; Highland Trail; 24 April 2006; Coll. D. Mausel |
| HM803394–HM803400 | 7 | *L. rubidus* | USA; VA; Giles County; Big Stony; 15 April 2006; Coll. D. Mausel |
| HM803434 | 1 | *L. rubidus* | USA; CT; Water County; Hamden; 27 March 2007; Coll. N. Havill |
| HM803471 | 1 | *L. rubidus* | USA; GA; Rabun County; Satolah; 26 March 2008; Coll. D. Mikus, J.C. Fagan |
| HM803473 | 1 | *L. rubidus* | USA; CT; Water County; Hamden; 17 April 2008; Coll. N. Havill, A. Serafin |
| HM803474–HM803477 | 4 | *L. rubidus* | USA; NC; Watauga County; Foscoe; 17 April 2008; Coll. M.E. Montgomery, R. McDonald |
| HM803478–HM803479 | 2 | *L. rubidus* | USA; CT; New Haven County; Woodbridge; 24 April 2008; Coll. V. Sanchez |
| HM803480–HM803486 | 7 | *L. rubidus* | USA; CT; New Haven County; Hamden; 25 April 2008; Coll. N. Havill, A. Serafin, M. Keena |
| HM803487–HM803490 | 4 | *L. rubidus* | USA; CT; New Haven County; Hamden; 30 April 2008; Coll. N. Havill, A. Serafin |
| HM803491 | 1 | *L. rubidus* | USA; MA; Hampden County; Holyoke; 5 May 2008; Coll. J. Biroscak |
| HM803492–HM803495 | 4 | *L. rubidus* | USA; CT; New Haven County; Wharton Brook State Park; 5 May 2008; Coll. M. Keena |
| HM803496–HM803504 | 9 | *L. rubidus* | USA; MA; Hampshire County; South Amherst; 6 May 2008; Coll. M.E. Montgomery |
| HM803591–HM803612 | 22 | *L. rubidus* | USA; PA; Center County; Boalsburg; 11 April 2009; Coll. N. Havill; M. Nehme |
| HM803613–HM803615, JF700523 | 4 | *L. rubidus* | USA; KY; Bell County; Yellow Creek; March 2009; R. Mays |
| HM803619–HM803657 | 39 | *L. rubidus* | USA; CT; Tolland County; Yale Meyers Forest; 17 April 2009; Coll. N. Havill, J. Klein |
| HM803658–HM803660, HM803679–HM803680 | 5 | *L. rubidus* | USA; MN; Crow Wing County; Eagle View Farms; 28 May 2009; Coll. N. Havill, J. Albers |
| HM803661 | 1 | *L. rubidus* | USA; MN; Beltrami County; Turtle River; 29 May 2009; Coll. N. Havill |
| HM803662–HM803668 | 7 | *L. rubidus* | USA; MN; Lake County; Gooseberry Falls State Park; 1 June 2009; Coll. N. Havill |
| HM803669–HM803671, HM803675–HM803678 | 7 | *L. rubidus* | USA; MN; Itasca County; UMN North Central Research and Outreach Center; 28 May 2009; Coll. N. Havill, J. Albers |
| HM803672 | 1 | *L. rubidus* | USA; MN; Stearns County; Birch Lakes State Forest; 30 May 2009; Coll. N. Havill, R. Tiplady |
| HM803673–HM803674 | 2 | *L. rubidus* | USA; MN; Itasca County; Bena; Portage Rd.; 28 May 2009; Coll. N. Havill |
| HM803686–HM803687 | 2 | *L. rubidus* | USA; TN; Blount County; Great Smoky Mountains National Park; 30 March 2007; Coll. G. Davis |

## 3. Results

### 3.1. DNA barcoding

*Laricobius* COI sequences were 702 bp long (658 with primers removed). Pairwise Kimura 2-parameter distances within *L. nigrinus* ranged from 0% to 1.23%, within *L. rubidus* from 0% to 1.07%, and between *L. nigrinus* and *L. rubidus* from 1.38% to 2.97%. The haplotype network (Fig. 1) indicates that there are eight diagnostic nucleotide sites separating the two species (Table 2). Of these, three are pure diagnostics with fixed substitutions in both species. The rest are fixed in one species while the other species has predominantly a different base with the exception of rare haplotypes.

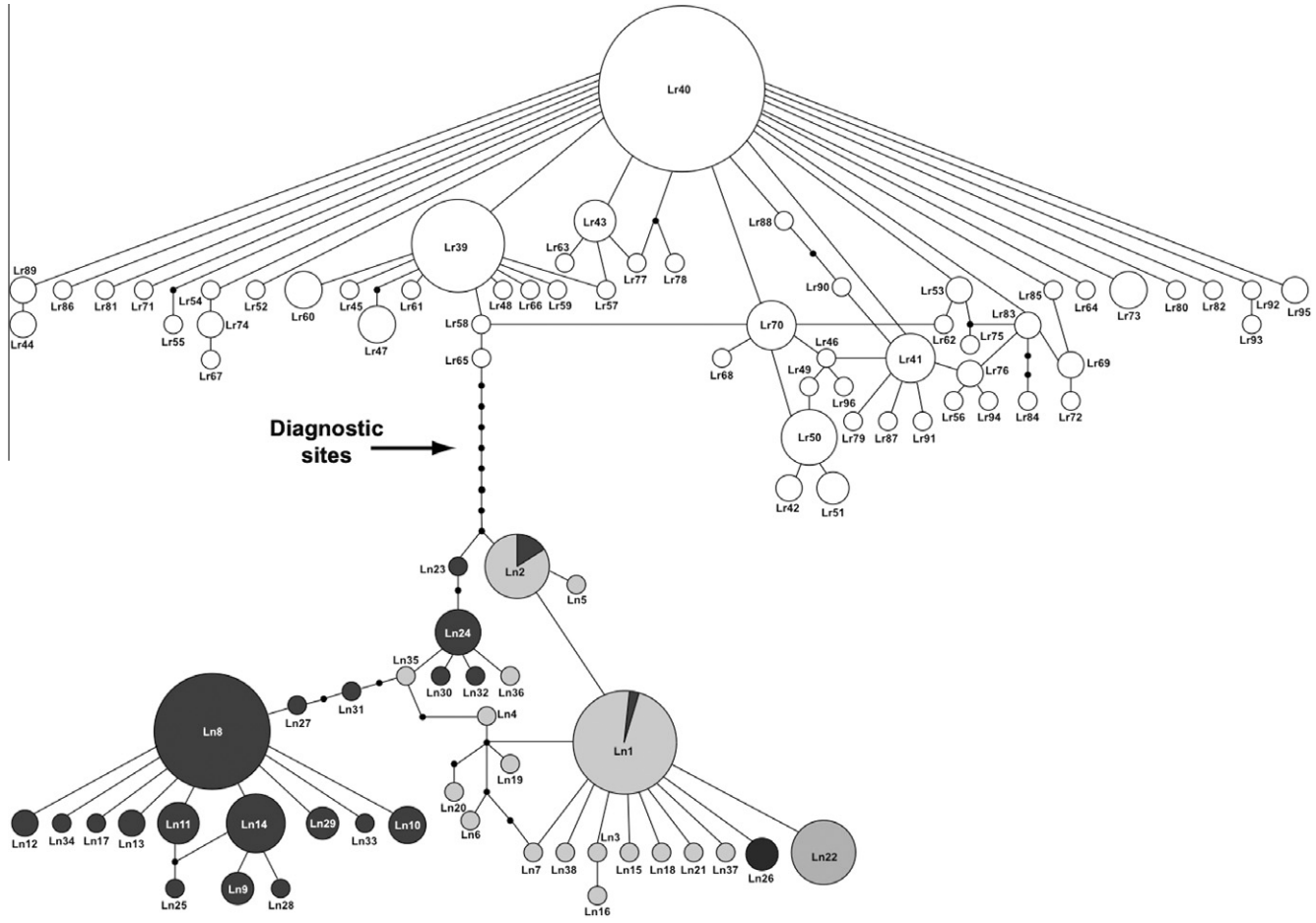


**Fig. 1.** Network showing relationships among COI haplotypes for *L. nigrinus* and *L. rubidus* samples. The size of each circle is proportional to the frequency of the haplotype. Haplotype names are included in or next to circles. Small black circles represent mutational steps separating observed haplotypes. White circles are haplotypes from *L. rubidus* samples, dark gray are coastal *L. nigrinus* collected from Washington, Oregon, and British Columbia, light gray are interior *L. nigrinus* from Idaho and Montana. The eight nucleotide changes that separate the species are indicated.

**Table 2**
Diagnostic characters to distinguish *L. nigrinus* from *L. rubidus*. Results are based on 658 base pairs of the mitochondrial COI gene sequenced from 157 *L. nigrinus* individuals (39 unique haplotypes), and 205 *L. rubidus* (59 haplotypes). Nucleotide position is based on the amplified region with primer sequences removed. For polymorphic positions within species, the number of haplotypes with each nucleotide is shown in parentheses.

| | Nucleotide position | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 58 | 190 | 223 | 274 | 475 | 542 | 619 | 658 |
| *L. nigrinus* | T | A | T | A | T | C (38)<br>T (1) | A | T |
| *L. rubidus* | A | G (57)<br>A (1)<br>T (1) | C | G (57)<br>A (1)<br>C (1) | C (54)<br>T (4)<br>A (1) | T | G (57)<br>A (2) | C |

### 3.2. PCR–RFLP

Digestion by AseI produced four fragments for *L. rubidus* of 231, 221, 169, and 81 bp, and three fragments for *L. nigrinus* of 313, 220, and 169 bp. Digestion with HpaII resulted in two fragments of 362 and 340 bp for *L. nigrinus*. Two fragment patterns resulted for *L. rubidus* cleaved by HpaII. The more common pattern had five fragments of 234, 156, 143, 106, and 63 bp, while the less common had four fragments of 340, 156, 143, and 63 bp (Fig. 2). All samples of *L. nigrinus* were correctly identified with both enzymes. All *L. rubidus* samples were correctly identified with HpaII and all but one sample was identified with AseI. The exception (HM803633) was not visually distinguishable from *L. nigrinus* fragments. Sequence data revealed a transition (T:C) at DNA barcode position 59 that prevented an additional AseI cleavage, which resulted in fragment sizes 312, 221, and 169 bp.

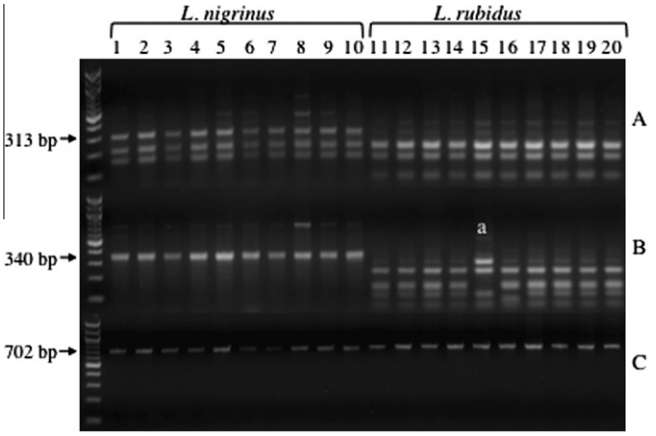


**Fig. 2.** Agarose gel electrophoreses of restriction fragments following PCR for *L. nigrinus* (lanes 1–10), *L. rubidus* (lanes 11–20), and 100 bp ladder. (A) AseI-cleaved fragments, (B) HpaII-cleaved fragments, (C) PCR amplified products prior to digestion. [a]Less common fragment size cut by HpaII.

### 3.3. qPCR

A 117 bp region of COI amplified for all 40 samples (Fig. 3). All *L. rubidus* samples were accurately determined by the increase of FAM fluorescence, with an average Cq of 24.6 ± SE 0.46 and a range from 19.5 to 31.4. The Cq or cycle threshold is the amplification cycle when a fluorescence signal is detected above a fixed threshold set within the software program. Only 11 of the 20 *L. nigrinus* samples were determined by this assay. *L. nigrinus* samples with adequate probe hybridization and subsequent release of VIC had an average Cq of 22.8 ± SE 0.55 and a range from 18.9 to 27.4. Based on these results, upper Cq values of 32 and 28 were set for acceptable discrimination of *L. rubidus* and *L. nigrinus*, respectively. Samples with Cq values above the threshold were undetermined. Seven of the undetermined *L. nigrinus* samples were collected in Idaho (HM803547–HM803551 and HM803553–HM803554) and the remaining two collected in Washington (HM803574 and HM803575). Undetermined calls by StepOne indicate a lack of probe hybridization with the target sequence. Only hybridized probes cleaved from the target during the extension phase release the 5′-fluorescent reporter protein away from the 3′-quencher molecule, allowing signal emittance. Sequence data for the Idaho samples exposed a transition at DNA barcode position 217 (Probe = G, sample sequence = A), while the two undetermined samples from Washington contained three transitions at barcode nucleotide positions 214 (A:G), 217 (G:A), and 226 (T:C). An additional sample from Washington (HM803573) contained transition changes at DNA barcode positions 217 (G:A) and 226 (T:C), yet produced a Cq of 27.0. The assay had reliable sensitivity in distinguishing *L. nigrinus* and *L. rubidus*. Diagnostic specificity was realized for all *L. rubidus* samples and slightly reduced to 95% for *L. nigrinus*.

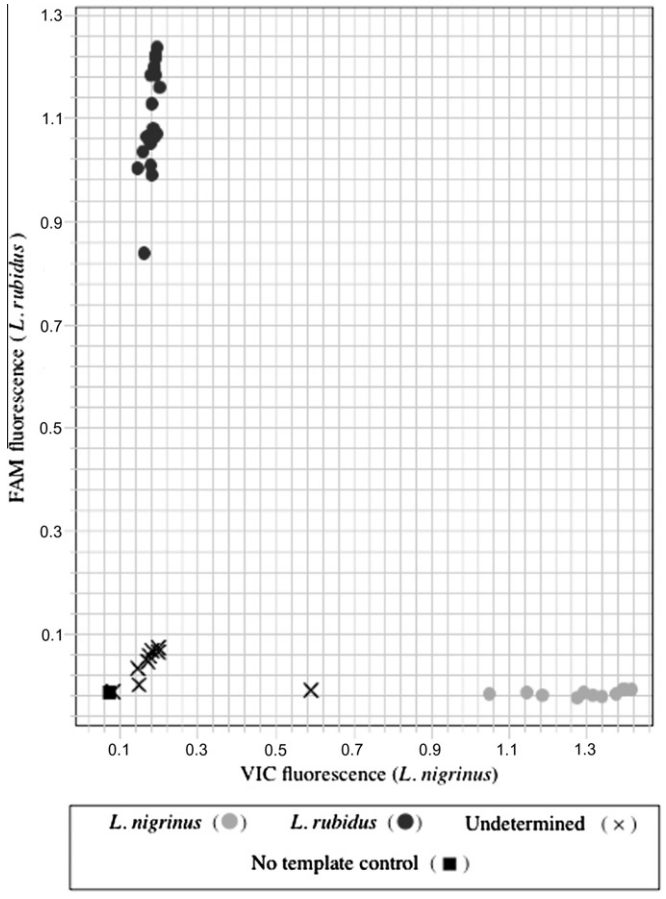


**Fig. 3.** Real-time PCR allele discrimination plot for the 20 samples of each *L. nigrinus* and *L. rubidus*.

### 3.4. Prey species

*A. tsugae* and *P. strobi* sequences were 702 bp long (658 with primers removed). Banding patterns after restriction enzyme digestion were different than those for *Laricobius*. PCR products of *A. tsugae* digested with AseI produced six fragments of sizes 144, 132, 113, 109, 106, and 98 bp, and *P. strobi* products produced seven fragments of size 230, 144, 106, 101, 81, 28, and 12 bp. Digestion with HpaII produced two fragments of sizes 643 and 59 for *A. tsugae* and there were no cut sites for *P. strobi*. qPCR alleic discrimination resulted in undetermined calls for *A. tsugae* and *P. strobi* and Cq values of 16.8 for *L. nigrinus* and 17.5 for *L. rubidus*. Examination of sequence data confirmed that the TaqMan assay primers for *L. nigrinus* and *L. rubidus* do not complement *A. tsugae* and *P. strobi* sequences.

## 4. Discussion

Efforts were taken to archive DNA barcodes for specimens from a wide geographic range for both *L. nigrinus* and *L. rubidus*. Species-specific DNA barcodes representing several geographic populations provide a valuable reference to identify unknown specimen sequences within taxa (Greenstone et al., 2005; Wilson and Schiff, 2010). PCR-based molecular diagnostics are increasingly applied to morphologically indistinguishable specimens (Weathersbee et al., 2004; Traugott et al., 2006; Ståhls et al., 2009). The two described PCR methods provide options to distinguish *L. nigrinus* and *L. rubidus*.

Two scenarios demonstrate how application needs changed or revealed additional information that led to the subsequent reevaluation of methods for accuracy and relevance. Both assays were designed to target diagnostic nucleotide changes using fewer reference specimens than we report here. PCR–RFLP was first used to quantify *L. nigrinus* dispersal and resulted in the discovery of a rare, but distinct third banding pattern for *L. rubidus* (HpaII fragment sizes 340, 299, and 63 bp). Those individuals were later confirmed to group with *L. rubidus* DNA barcodes.

The second scenario involved changes in biological control management strategies for *L. nigrinus*. Before 2008, all *L. nigrinus* introductions into the eastern United States were from coastal populations in western North America. The need for more cold-tolerant *L. nigrinus* in the northeastern range of *A. tsugae* became apparent in 2008. To address this, a biotype from an inland population in western North America was introduced to the northeastern United States (Mausel et al., 2009). The haplotypes network (Fig. 1) shows that coastal and interior populations cannot be distinguished 100% of the time with COI because they lack diagnostic sites that are fixed in each group. This may be due to incomplete lineage sorting or continued gene flow between the groups. Future work using microsatellites will examine this pattern further.

While both *L. nigrinus* populations were distinguished from *L. rubidus* using PCR–RFLP, this was not the case with the qPCR assay. Hydrolysis probes used in the qPCR assay were designed from sequences within the coastal *L. nigrinus* populations. The assay successfully distinguished over 700 *Laricobius* larvae collected from locations where the coastal *L. nigrinus* haplotypes were introduced to the eastern United States (G. Davis, unpublished data). Assay results were confirmed as 99% accurate through sequence analysis of a portion of the COI gene and several nuclear loci (Havill et al., 2010). Yet, probe specificity of the same assay prevented hybridization with DNA of the seven *L. nigrinus* samples of the inland (Idaho) population and two samples from Washington due to base

changes in the targeted region of the template. Redesigning the qPCR primers and probes could create an assay able to discriminate species regardless of the 98 distinct haplotypes between the congeners. Alternatively, it may be more practical to design assays based upon geographic region. For example, from the mid-Atlantic to the southern range of the exotic *A. tsugae* distribution, only coastal populations of *L. nigrinus* have been introduced. These introduction sites could be accurately monitored using the described assay.

Regardless of assay design and optimization of thermocycling conditions, mismatched probe hybridizations can occur, as observed for one sample from Washington (HM803573). Yao et al. (2006) reported MGB probe hybridization in the presence of two mismatched nucleotides and recorded fluorescence signal at a higher Cq. Fluctuation of Cq levels can also result from variable DNA purity and reduce quantification sensitivity. These factors should be considered when analyzing qPCR results.

A molecular phylogeny using COI and three nuclear regions that included five additional *Laricobius* confirmed that *L. nigrinus* and *L. rubidus* are recently diverged sister species (Montgomery et al., 2011). This raised the possibility that *L. nigrinus* and *L. rubidus* could successfully hybridize in the field, and in fact, hybrid offspring have been recently confirmed at three *L. nigrinus* introduction sites (Havill et al., 2010). It should be noted that the assays described here would not detect hybrid individuals because the COI mitochondrial gene is strictly maternally inherited. Therefore, these assays only track an individual's maternal lineage. If hybrids were sampled, the two PCR assays would still be valuable for determining the presence and persistence of *L. nigrinus* populations where female *L. nigrinus* reproduce. Hybrid detection requires additional analyses such as one based on fast-evolving nuclear markers described by Klein et al. (2010).

The specificity of the PCR–RFLP and qPCR assays would prevent the misclassification of *L. nigrinus* or *L. rubidus* due to amplification of prey DNA present in their guts. It is likely that adelgid DNA was present in some *Laricobius* PCR reactions because DNA was extracted from beetle abdomens, and beetles were not starved prior to extraction. COI successfully amplified for *A. tsugae* and *P. strobi* using the universal DNA barcode primers, but the downstream steps in both assays are specific to *Laricobius*. For the PCR–RFLP assay, the number and size of fragments after digestion produced distinct patterns for predators versus prey when visualized on an agarose gel. The prey-specific banding patterns were not seen in any of the gels suggesting that *Laricobius* DNA was preferentially amplified. Primers that are specific to adelgid species could be incorporated into the PCR–RFLP assay to detect the presence of prey in *Laricobius* guts (e.g. Hosseini et al., 2011). For the qPCR assay, the combination of specific primers and probes provide the desired level of specificity. This is commonly done to avoid misclassification of non-target organisms (e.g. Fitzpatrick and Caron, 2010; Vrba et al., 2010).

The availability of DNA barcodes and the described PCR methods serve a current need of universities, government agencies, and non-government cooperators that are monitoring *L. nigrinus* introduction sites to determine species establishment, dispersal, and potential non-target effects. Choosing a method for species discrimination will depend on resources and available equipment. Equipment required for PCR–RFLP is standard for most molecular labs, while a more costly fluorescence detecting thermocycler is required for qPCR. Generally, the per-reaction cost of qPCR reagents is greater than PCR–RFLP. We found qPCR increased sample processing efficiency 10-fold over PCR–RFLP. Feng et al. (2007) suggested qPCR reduced processing by 3–7 h over PCR–RFLP. The large reduction in lab hours offsets the higher reagent costs. Such a significant reduction in processing time may allow for more extensive sampling strategies and hence greater statistical power in evaluating biological control success.

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