HEMLOCK WOOLLY ADELGID BIOLOGICAL CONTROL: MOLECULAR METHODS TO DISTINGUISH *LARICOBIUS NIGRINUS*, *L. RUBIDUS*, AND THEIR HYBRIDS

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ABSTRACT

Molecular diagnostics use DNA-based methods to assign unknown organisms to species. As such, they rely on a priori species designation by taxonomists and require validation with enough samples to capture the variation within species for accurately selecting diagnostic characters. Molecular diagnostics are increasingly recognized as powerful tools to facilitate biological control programs. They can provide reliable and inexpensive identification of natural enemies, track their population dynamics, and monitor impacts on target and non-target organisms. We report preliminary results using DNA-based methods to distinguish *Laricobius nigrinus* (Coleoptera: Derodontidae) from *L. rubidus*, which is native to eastern North America where it is commonly associated with pine bark adelgid, *Pineus strobi* (Hemiptera: Adelgidae). *Laricobius rubidus* also feeds on the introduced hemlock woolly adelgid, *Adelges tsugae*, but has not been shown to impact its populations. *Laricobius nigrinus* is native to western North America and has been released as a biological control agent of *A. tsugae* in multiple locations in eastern North America where *L. rubidus* occurs. The two species can be distinguished as adults by color and by differences in male genitalia. *Laricobius* larvae cannot be readily distinguished using morphology; rearing them to the adult stage for identification is labor intensive, and many beetles die in the process. In addition, *L. nigrinus* and *L. rubidus* have been observed mating in the field (Mausel et al. 2008) and were found to be closely related sister species (Montgomery et al. in prep.), which has drawn attention to the possibility of successful hybridization.

We used three molecular approaches to address these identification and hybridization issues. The first method we used to monitor post-release establishment and spread of *L. nigrinus* was polymerase chain reaction, restriction fragment length polymorphism (PCR-RFLP). This method uses restriction enzymes to cleave DNA at sites with specific short nucleotide sequences. Restriction enzymes were chosen that target four diagnostic nucleotide positions in the mitochondrial cytochrome oxidase I (COI) gene that reliably differ between the species. Following PCR, restriction enzymes are used to cut the DNA into different sized fragments. This results in different banding patterns for each species when resolved by gel electrophoresis (Fig. 1). It should be noted that because COI is a mitochondrial gene, this assay identifies only an individual's maternal lineage and therefore cannot detect hybrids.

The second method we developed is a real-time PCR assay that amplifies a 117-base pair portion of COI and uses unique 20-nucleotide TaqMan® probes (Applied Biosystems). Separate probes were designed to bind specifically to *L. nigrinus* or *L. rubidus* and were tagged with different colored dyes. During amplification, the dye associated with the

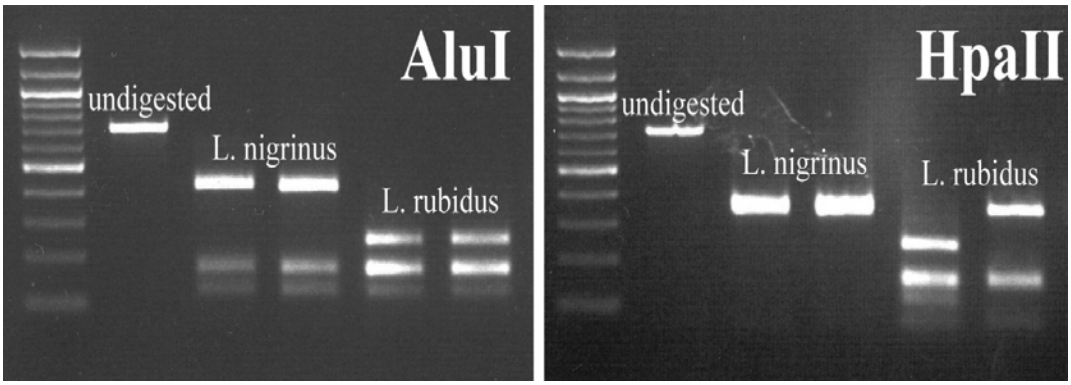

Figure 1.—Results of PCR-RFLP using the restriction enzymes AluI and HpaII to digest a 702-base portion of the COI gene. For each gel, the first lane is a 100-base pair DNA ladder (Invitrogen), the second lane is uncut COI PCR product, lanes 3 and 4 are *L. nigrinus*, and lanes 5 and 6 are *L. rubidus*. Note the two banding patterns for *L. rubidus* using HpaII.

beetle species' DNA that is present is released and detected (Fig. 2). To our knowledge, the application of TaqMan® real-time PCR to distinguish cryptic species is novel to biological control. The per reaction cost of real-time PCR reagents is greater than PCR-RFLP, but the second method is less time consuming.

Finally, we developed microsatellite markers to address whether *L. nigrinus* and *L. rubidus* have successfully hybridized in the eastern United States. Microsatellites are nuclear, co-dominant population genetic markers that, in contrast to mitochondrial DNA, provide information about both parental lines. Ten polymorphic

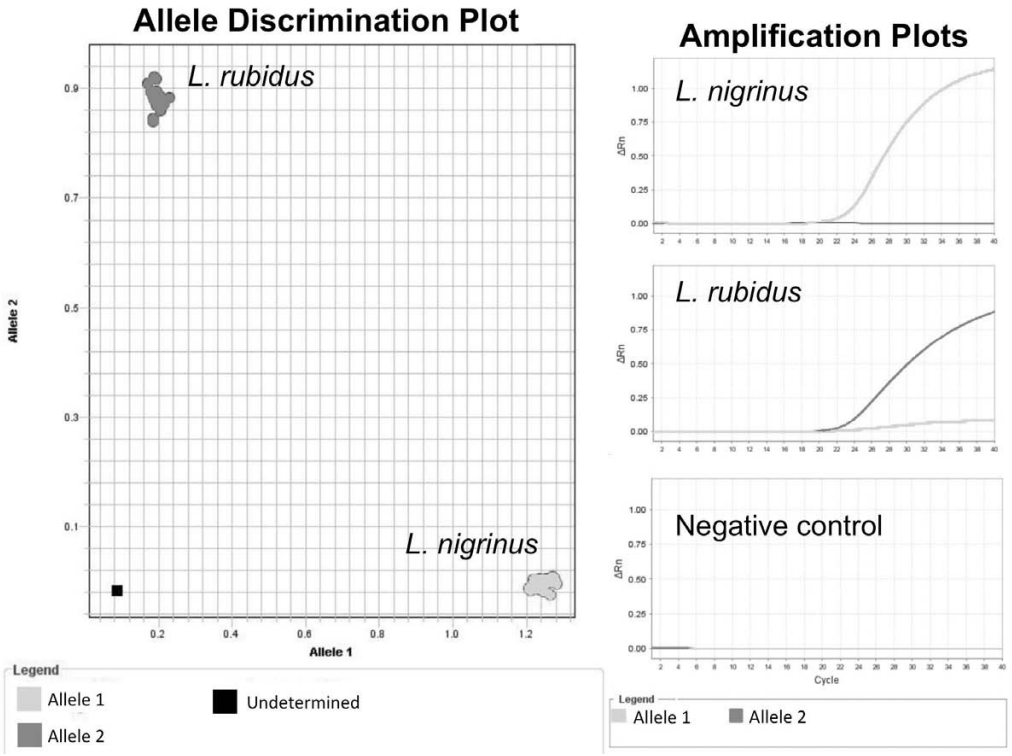

Figure 2.—Left: Real-time PCR allelic discrimination plot for samples of *L. nigrinus* (allele 1), *L. rubidus* (allele 2), and negative controls (black square). Right: Amplification plots showing detection of different dyes for each species.

dinucleotide microsatellite markers were isolated and characterized for both beetle species (Klein et al. in press). Six of these were used to genotype *Laricobius* larvae collected from sites with known *L. nigrinus* release dates. To date, we have genotyped larvae from sites in Tennessee and Pennsylvania (*L. nigrinus* released in 2004) and in North Carolina (*L. nigrinus* released in 2005). We found clear evidence of hybridization at all three sites, with backcrosses in both directions (example from Tennessee is shown in Figure 3). The consequences of hybridization between *L. nigrinus* and *L. rubidus* for adelgid biological control are not known. Hybrid offspring could be more fit than their parents (hybrid vigor), which could enhance biological control but result in homogenization of the two species; or, hybrids could be less fit (outbreeding depression or hybrid sterility), which could ultimately maintain species integrity but could impair control efforts. Introgression of genes between species could also change their host preferences. Future work will evaluate the impacts of hybridization on the efficacy of adelgid biological control.

Accurate species determination is vital to biological control efforts. PCR-based molecular diagnostics can provide rapid and inexpensive identification when morphological characters are not sufficient. The combination of PCR-RFLP, real-time PCR, and microsatellites has proven to be very useful for understanding the establishment and spread of *L. nigrinus.*

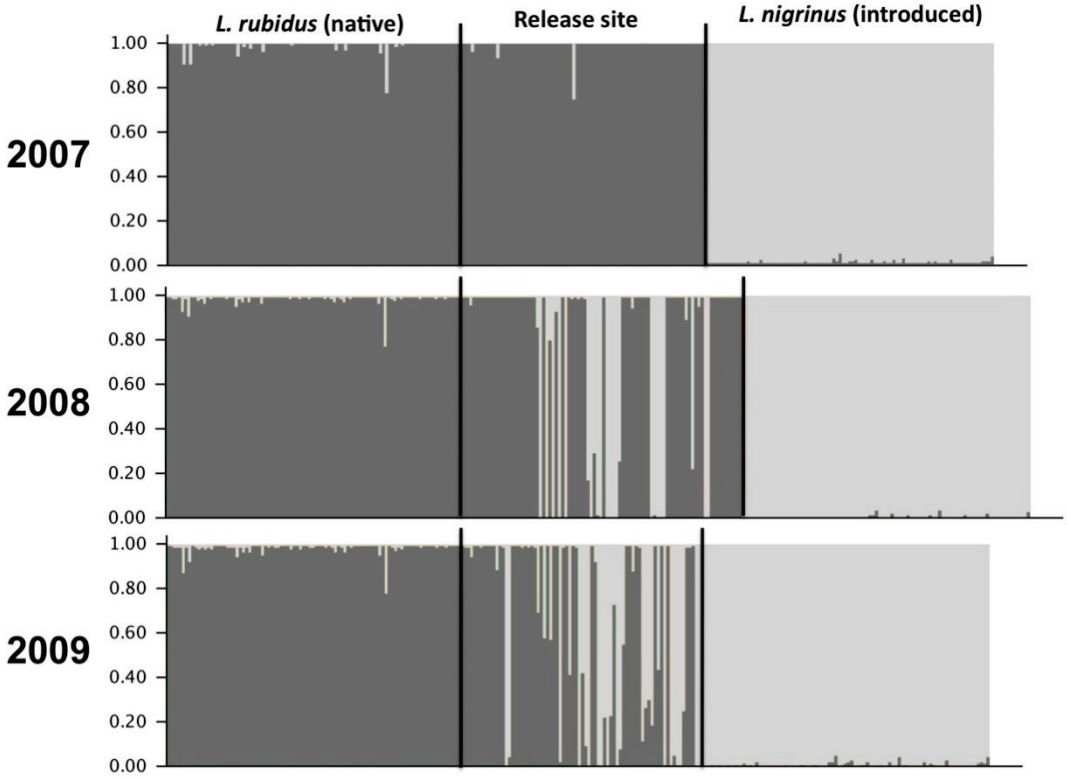

Figure 3—The chart shows the probability of assignment (q; y-axis) to L rubidus (dark gray) or *L. nigrinus* light gray) for individual beetles (x-axis). Data are from six microsatellite loci and are analyzed using the software Structure (Pritchard et al. 2000). Reference samples of 248 *L. nigrinus* from Washington, Oregon, Idaho, and British Columbia are on the right, and 164 *L. rubidus* from non-release sites in Connecticut, Massachusetts, Kentucky, Pennsylvania, and Minnesota are on the left. *Laricobius* larvae collected over 3 years from a site in the Great Smoky Mountains National Park in Tennessee where *L. nigrinus* was released in 2004 are in the center. For the release site, an increase in the mean probability of assignment to *L. nigrinus* (light gray) over time indicates that the proportion of pure *nigrinus* and its hybrids is increasing (2007: q=0.01, 2008: q=0.29, 2009a; q=0.41).

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