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Hybridization between a native and introduced predator of Adelgidae: An unintended result of classical biological control

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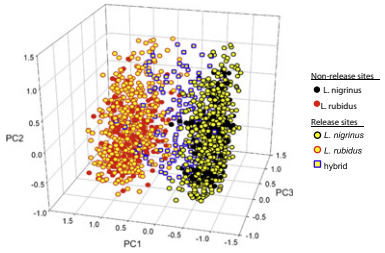
HIGHLIGHTS

- We report hybridization between *Laricobius nigrinus* and *Laricobius rubidus*.
- Six microsatellite markers plus mitochondrial COI haplotypes were used.
- Introgression was widespread and asymmetrical towards *L. nigrinus*.
- The outcome could be a mosaic of genetic introgression across the landscape.
- Evaluating hybridization between biocontrol agents and native species is important.

GRAPHICAL ABSTRACT



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ABSTRACT

Hybridization between introduced biological control agents and native species has the potential to impact native biodiversity and pest control efforts. This study reports progress towards predicting the outcome of hybridization between two beetle species, the introduced *Laricobius nigrinus* Fender and the native *L. rubidus* LeConte. *L. nigrinus* is a predator from western North America introduced to hemlock stands in the eastern United States as a biological control of the hemlock woolly adelgid [*Adelges tsugae* Annand (Hemiptera: Adelgidae)]. *Laricobius rubidus* is a closely related eastern species that also feeds on *A. tsugae* but prefers pine adelgids (*Pineus strobi* Hartig) on white pine (*Pinus strobus* L.). Six microsatellite markers plus mitochondrial COI haplotypes were used to examine genetic structure of these two *Laricobius* species across North America. In their native ranges, major geographic features have impacted gene flow: the intermountain region in the West, and the Appalachian Mountains in the East. Analysis of 1229 individuals from adelgid-infested hemlock trees in release sites in the eastern United States found widespread hybridization with asymmetrical introgression towards *L. nigrinus* on hemlock. The ultimate outcome of hybridization could therefore be a complex mosaic of genetic introgression across the landscape, depending on the distribution of hemlock and pine. This study confirms the importance of evaluating the potential for introduced biological control agents to hybridize with their native relatives. This system also provides an excellent opportunity to improve our understanding of emerging hybrid zones by tracking its progress over time.

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1. Introduction

Hybridization is widespread in nature with complex ecological and evolutionary dynamics (Dobzhansky, 1940; Hewitt, 1988; Harrison, 1993; Burke and Arnold, 2001). Factors that influence whether hybridizing species ultimately merge or remain distinct include the permeability of reproductive barriers and the strength of selection against hybrids. Additional complexity is introduced when fitness is differentially impacted by environmental factors. Together, this makes it very difficult to predict the outcome of any particular hybridization event.

Accidental or deliberate introduction of non-native species that leads to hybridization with native species is a conservation concern (Rhymer and Simberloff, 1996; Allendorf et al., 2001; Mooney and Cleland, 2001; Seehausen et al., 2008; Ellstrand et al., 2010). Hybridization between introduced biological control agents and native species has also been discussed in this context because of potential impacts on native biodiversity or on the success of pest control (Hopper et al., 2006; van Lenteren et al., 2006).

We are aware of just three cases of hybridization between a classical biological control agent and a native species. *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) is used for biological control of greenhouse pests. Naka et al. (2005, 2006) were able to produce laboratory crosses between *C. carnea* introduced from Germany, and native Japanese *C. nipponensis* (Okamoto). In this case, hybridization was not expected to threaten the integrity of the native species because hybrid fertility was low, and each parent species has a different courtship song. The second example involves *Diadegma semiclausum* (Hellen) (Hymenoptera: Ichneumonidae), a parasitoid used to control diamondback moth, which was shown to hybridize with native Japanese *D. fenestrale* (Holmgren) in the lab (Davies et al., 2009). Field evaluations have not been reported. Finally, *Torymus sinensis* Kamijo (Hymenoptera: Torymidae), introduced from China, was found to hybridize with native Japanese *T. beneficus* Yasumatsu et Kamijo in the lab (Moriya et al., 1992) and in the field (Toda et al., 2000; Yara et al., 2000). The native *Torymus* species has separate early-spring, and late-spring strains. The introduced species displaced the early-spring strain, without evidence of hybridization, while an increasing proportion of hybrids with the late-spring strain was detected over time (Yara et al., 2010). Release of *T. sinensis* resulted in successful control of the pest species, but the specific effects of hybridization on biological control were not evaluated.

Based on these few examples, it is not possible to predict the impact of hybridization on a particular biological control program. This requires specific information about the extent of interbreeding and the relative fitness of hybrids versus parents in the ecological context in which they are likely to be found. Hybrids can be less fit as expressed by outbreeding depression or hybrid sterility (Dobzhansky, 1940), which could maintain species integrity but impair control efforts if hybrids have less of an impact on the pest than their parents. Conversely, hybridization can be a source of novel genetic variation, introducing advantageous alleles or allele combinations that can contribute to adaptive evolution (Lewontin and Birch, 1966). This could enhance establishment and efficacy of the biological control agents but disrupt the genetic integrity of the native species with cascading effects on its own predator–prey dynamics.

The hemlock woolly adelgid, *Adelges tsugae* Annand, was introduced from Japan to the eastern United States (Havill et al., 2006) some time before its discovery in Virginia in the early 1950s. It has become a serious pest of eastern hemlock, *Tsuga canadensis* L. (Carrière) and Carolina hemlock, *T. caroliniana* Engleman. In western North America there is a native lineage of *A. tsugae* (Havill et al., 2007) that has a suite of predators that may be instrumental in regulating its populations (Kohler et al., 2008). Western North America has therefore been considered a promising source of biological control agents for introduction to the eastern United States.

One species, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), has been released since 2003 throughout the introduced range of *A. tsugae* in the eastern United States (Mausel et al., 2010). It has become established at many of the release sites and has been shown to reduce adelgid densities in the field, but the recovery of hemlock forest health has not been documented to date (Mausel et al., 2008, 2011b). *Laricobius* species are known to feed only on Adelgidae (Leschen, 2011). In laboratory tests, *L. nigrinus* preferred feeding and ovipositing on *A. tsugae* settled on *T. canadensis* over the native eastern pine bark adelgid, *Pineus strobi* (Hartig) settled on *Pinus strobus* L., and *L. nigrinus* completed development only on *A. tsugae* (Zilahi-Balogh et al., 2002). In western North America, *L. nigrinus* has been collected from *Tsuga heterophylla* (Raf.) Sarg., *Pseudotsuga menziesii* (Mirb.) Franco, *Picea engelmannii* Parry ex Engelm., *Pinus monticola* Douglas ex D. Don (Mausel et al., 2011b), and *Larix occidentalis* Nutt. (this study).

Laricobius rubidus LeConte is the only member of the genus that is endemic to eastern North America (Leschen, 2011). It is most commonly associated with *Pineus strobi* on *Pinus strobus* (Clark and Brown, 1960), but has also been reported feeding on three non-native adelgids: *A. tsugae* on *T. canadensis* (Montgomery and Lyon, 1996; Wallace and Hain, 2000; Mausel et al., 2008), *Adelges piceae* Ratz., on *Abies* spp. (Clark and Brown, 1960), and *Pineus pini* (Macquart) on *Pinus sylvestris* L. (this study). In laboratory tests, *L. rubidus* developed and survived equally well when reared on *A. tsugae* or *P. strobi*, but preferred to oviposit on *P. strobi* (Zilahi-Balogh et al., 2005). The two *Laricobius* species can be distinguished as adults by the color of their elytra and by differences in the shape of male genitalia (Leschen, 2011; Montgomery et al., 2011). Larvae of the two species cannot be distinguished morphologically.

Davis et al. (2011) evaluated genetic diversity within and differentiation between *L. nigrinus* and *L. rubidus* using a section of the cytochrome c oxidase subunit I (COI) mitochondrial gene commonly used for DNA barcoding. They found low sequence divergence between the two species. A phylogeny of the genus *Laricobius* reconstructed by Montgomery et al. (2011) confirmed that *L. nigrinus* and *L. rubidus* are recently diverged sister species. These results, plus observations of *L. nigrinus* and *L. rubidus* mating in the field (Mausel et al., 2008), drew attention to the possibility of hybridization between the two species.

The purpose of this study is to make progress towards predicting the impacts of hybridization between *L. nigrinus* and *L. rubidus* on biological control of *A. tsugae* and on native biodiversity in eastern North America. Microsatellite markers were evaluated for their ability to distinguish each species and their hybrids and were used to test for population structure within each species. Microsatellites and mitochondrial COI DNA sequence variation were then used to evaluate the incidence of hybridization in the field using samples collected on *T. canadensis* infested with *A. tsugae* at sites in the eastern United States where *L. nigrinus* was released. Adult beetle morphology was also examined to determine whether there are characters that can be used to distinguish parent species from hybrids.

2. Methods

2.1. Sample collection and data acquisition

Beetles were collected between 2006 and 2011 from 57 sites in the United States and one site in Canada (Fig. 1; Supplementary Table 1). Some of these specimens were also analyzed in Davis

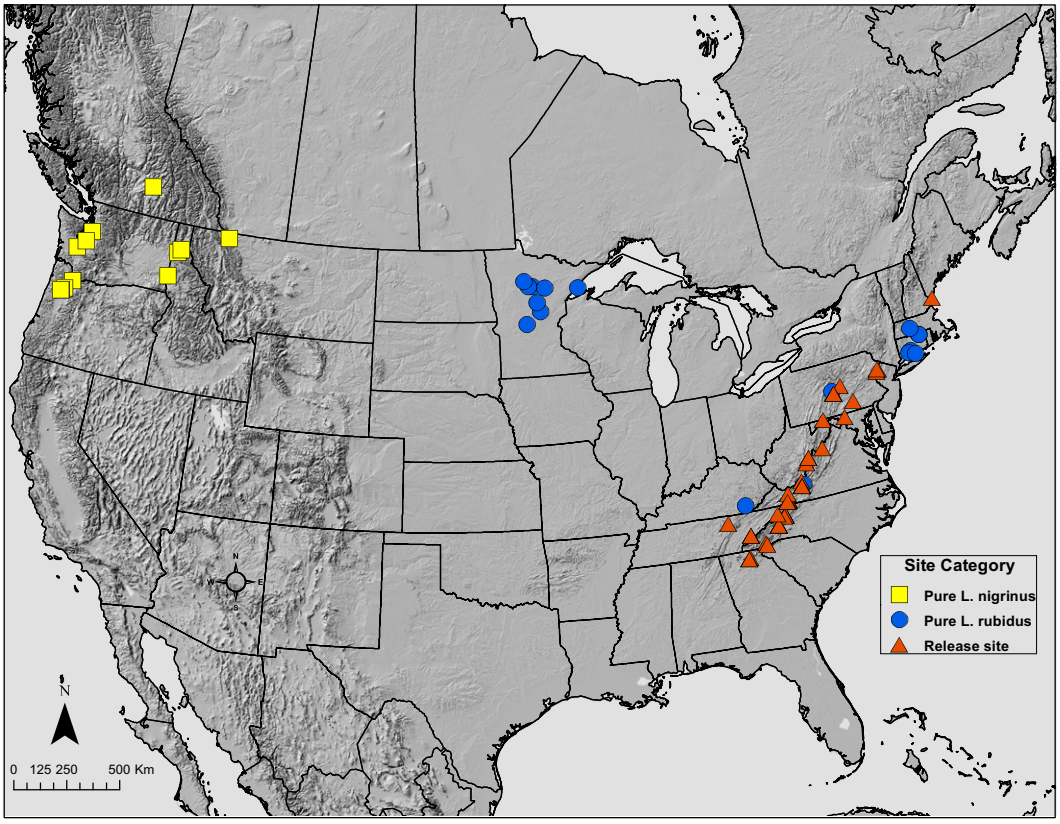

Fig. 1. Map showing collection sites.

et al. (2011). Samples of purebred *L. nigrinus* were collected in its native range in western North America from *Tsuga heterophylla* infested with *A. tsugae*, *Pinus monticola* infested with *Pineus* sp., or *Larix occidentalis* infested with *Adelges lariciatus* (Patch). Purebred *L. rubidus* were collected from sites in the eastern United States where *L. nigrinus* had not yet been released. They were collected from *Pinus strobus* infested with *Pineus strobi*, except for one sample from *Pinus sylvestris* infested with *Pineus pini*. Samples of unknown genetic ancestry were collected from sites where *L. nigrinus* had been released in the native range of *L. rubidus*. Adults were collected using beat sheets from lower branches of trees infested with adelgids. Larvae were collected by examining adelgid-infested branches under a dissecting microscope, or by cutting branches and placing them in modified Burlese funnels, where larvae developed to the pre-pupal stage then dropped into collection containers (Lamb et al., 2005). Beetles were preserved in 95% ethanol and stored at −20 °C until DNA extraction. Abdomens of adults were removed, while the head, thorax, and elytra were kept as vouchers. Male genitalia were slide-mounted in Canada balsam. Abdomens of larvae were removed with a scalpel and the head and thorax were slide mounted in Canada balsam as a voucher. Genomic DNA was isolated from beetle abdomens with the DNA IQ Extraction Kit (Promega). Vouchers were deposited at the Peabody Museum of Natural History at Yale University.

Six nuclear microsatellite loci (LaGT01, LaCA04, LaGT07, LaGT13, LaCA14, LaCA16) that were highly variable and relatively straightforward to score were used to genotype all beetle samples. Microsatellite loci were amplified via Polymerase Chain Reaction (PCR) in 12.5 μL volumes using the conditions described in Molecular Ecology Resources Primer Development Consortium et al. (2010). PCR products were analyzed using an ABI 3730 automated sequencer at the DNA Analysis Facility on Science Hill at Yale University, New Haven, CT, and genotypes were scored using Genemapper 4.0 (Applied Biosystems). Only samples with complete microsatellite genotypes were used for subsequent analyses. Mitochondrial COI haplotypes were scored binomially for genotypes diagnostic for *L. nigrinus* or *L. rubidus* using HpaII restriction fragment length polymorphism patterns or DNA sequences as described in Davis et al. (2011). All new COI sequences generated for this study were deposited in GenBank (accession numbers JX412482–JX412723).

2.2. Population structure within and between species

Arlequin 3.5.1.3 (Excoffier et al., 2005) was used to calculate diversity indices and test for Hardy–Weinberg equilibrium and differentiation (F_{ST}) among species and populations using the infinite-allele model and 1000 permutations. Bonferroni corrections were applied to *p*-values for multiple comparisons. For comparisons among populations within species, samples were pooled across years and among sites that were less than 50 km apart. Only populations with 12 or more samples after pooling were included. To test for isolation by distance, IBDWS 3.23 (Jensen et al., 2005) was used to perform Mantel tests (with 1000 permutations) of the correlation between genetic differentiation and geographic distance among populations for each species. Partial Mantel tests were used to test for correlation between the genetic distance and association with the major mountain range in the native range of each species, controlling for the effects of geographic distance.

To detect population structure within *L. nigrinus*, we analyzed 198 samples collected in its native range from 11 sites in Washington, Oregon, British Columbia, Idaho, and Montana. For *L. rubidus* an iterative approach was used. First, we analyzed a data set with 161 known *L. rubidus* samples collected from 17 sites where *L. nigrinus* had not been released in Minnesota, Connecticut, Massachusetts, Pennsylvania, Kentucky, Virginia, and Georgia. A second analysis was completed using these samples, plus those genetically identified as purebred *L. rubidus* (see Results) from sites

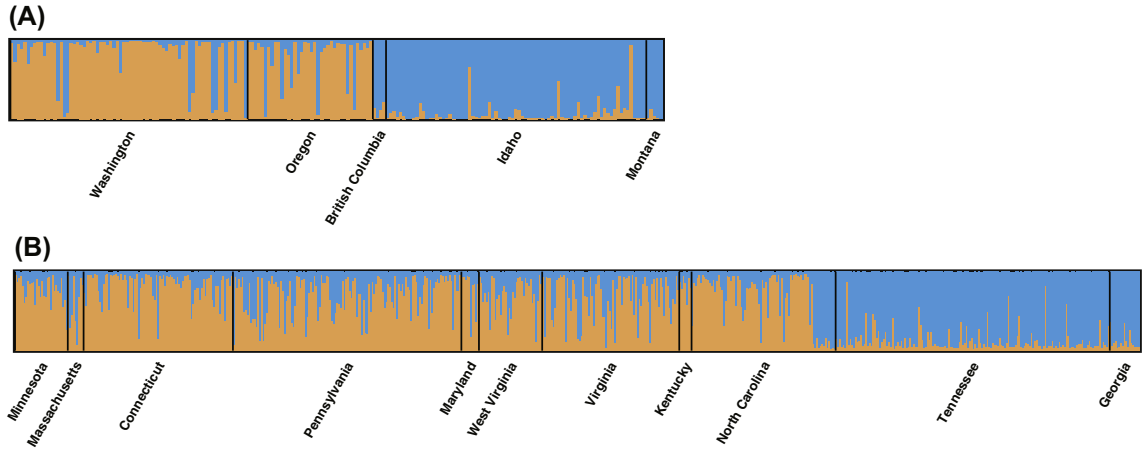

Fig. 2. Structure plots showing group membership within (a) *L. nigrinus* and (b) *L. rubidus*. Each individual is represented by a vertical bar partitioned into $K = 2$ colors. The height of each color corresponds to the probability of assignment to a different cluster. Vertical black lines separate individuals from different states or provinces. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

where *L. nigrinus* had been released. This second data set consisted of 640 samples.

Genetic structure between and within species was analyzed using the Bayesian clustering method implemented in Structure 2.3.2 (Pritchard et al., 2000). This method assigns individuals with probability q to a defined number of populations, K. All Structure runs used correlated allele frequency and an admixture model with 20,000 burn-in iterations followed by 100,000 sample iterations. To determine the optimal number of population clusters in a sample, we used the method of Evanno et al. (2005) calculated with Structure Harvester 0.6.8 (Earl and vonHoldt, 2012). Five independent runs were completed for each value of K from 1 to 8, and ΔK was calculated as the second-order rate of change between log probability values for successive values of K. The optimal number of populations was inferred from a spike in a plot of K versus ΔK. This method cannot recognize whether $K = 1$ is optimal (Evanno et al., 2005), so it is also necessary to consider whether $K = 1$ results in the highest likelihood value, and to examine the resulting Structure plots to determine whether the patterns make biological sense (Pritchard, 2007). Clumpp 1.1.2 (Jakobsson and Rosenberg, 2007) was used to combine results from five runs with the resulting optimal value of K. Graphical displays were created using Distruct 1.1 (Rosenberg, 2004). All subsequent Struscture runs used these same general parameters.

2.3. Evaluation of markers to distinguish hybrids from parent species

Simulated hybrid genotypes were generated to test the efficiency of microsatellite markers for distinguishing among *L. nigrinus*, *L. rubidus*, and their hybrids. Data from the samples of known *L. nigrinus* ($n = 198$) and *L. rubidus* ($n = 161$) were used as source populations to generate simulated genotypes using Hybridlab 1.0 (Nielsen et al., 2006). One hundred each of pure *L. nigrinus*, pure *L. rubidus*, F1 hybrids, F2 hybrids, *L. nigrinus* backcrosses, and *L. rubidus* backcrosses were simulated. A data set that included the 359 known samples, plus the 600 simulated samples was analyzed with Structure and Newhybrids 1.1 (Anderson and Thompson, 2002). Five independent Structure runs were combined, with $K = 2$, and with the known individuals coded as learning samples for updating allele frequencies. Newhybrids runs were completed with 10,000 burn-in iterations followed by 100,000 sample iterations, with individual-specific options used to identify the known individuals. Genotype frequency categories were included to represent each pure species, F1 hybrids, F2 hybrids, and each backcross. The results of five independent runs were averaged.

2.4. Estimating hybridization in L. nigrinus release sites

To identify the genetic ancestry of beetles in *L. nigrinus* release sites, a data set was compiled to include the samples of known *L. nigrinus* ($N = 198$), known *L. rubidus* ($N = 161$), and unknown samples from sites where *L. nigrinus* was released ($N = 1229$). Structure runs were completed using $K = 2$ and with individuals of known ancestry coded as learning samples. Newhybrids runs were completed with individual-specific options to identify samples of known ancestry. The results of five runs were averaged. As a low number of loci reduces the accuracy of assignment to a specific hybrid class (Vähä and Primmer, 2006), we summed the results across all four classes to obtain the probability of assignment as a hybrid. To visualize the genetic differentiation among individuals, we performed a principal components analysis (PCA) using individual allele frequencies with GenoDive 2.0b22 (Meirmans and Van Tiernderen, 2004).

There were three steps to classifying an individual as *L. nigrinus*, *L. rubidus*, or a hybrid. First, an individual was provisionally assigned to a parental species if Structure analysis resulted in $q > 0.80$ for that species, or as a hybrid if $0.20 > q < 0.80$. This cutoff value was chosen based on the results of the simulations described above (see Section 3.2). Next, if the category with the highest probability of assignment from Newhybrids agreed with Structure, then that assignment was retained. If they did not agree, then the individual was assigned to the category with the higher probability of the two analyses. Finally, if the COI classification disagreed with the microsatellite classification of a pure species, then the individual was classified as a hybrid.

2.5. Morphological analyses

Adult beetles from Kentland Farm at Virginia Tech University in Blacksburg, VA were used to determine whether morphological characters can distinguish hybrids from pure species. This site was established in 2001 as a field insectary to rear *L. nigrinus* for redistribution as biological controls (Salom et al., 2011). It consists of 0.4 ha of cultivated hemlock trees infested with hemlock woolly adelgid and supplied with lab-reared *L. nigrinus* starting in 2003. There is a plantation of white pine infested with *P. strobi* adjacent to the insectary, making this a good site to explore interaction between *L. nigrinus* and *L. rubidus*.

Laricobius nigrinus is entirely black, while *L. rubidus* has bicolored red and black elytra (Leschen, 2011; Fig. 5). A single elytron was removed from each beetle and placed under a dissecting

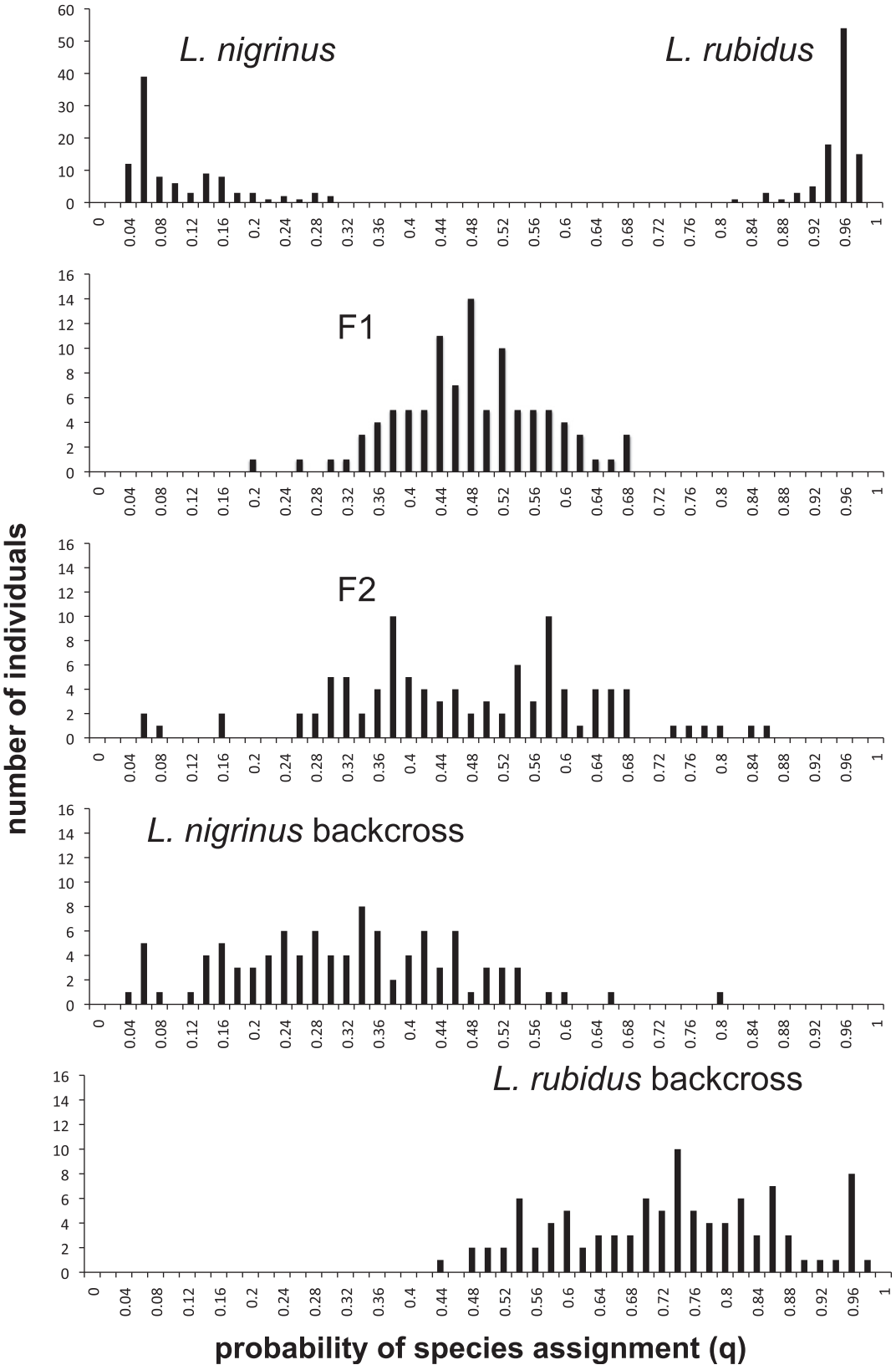

Fig. 3. Histograms of the probability of species assignment (q) resulting from analysis of population structure with $K = 2$ for simulated pure species and hybrid classes.

microscope on a frosted glass plate, illuminated from below, and digital images were acquired. The proportion of black on beetle elytra was measured with ImageJ (Rasband, 1997). First, the background color in the image was selected with the default thresholding method, B&W threshold color, and RGB color space. The background color was deleted to leave white space around the

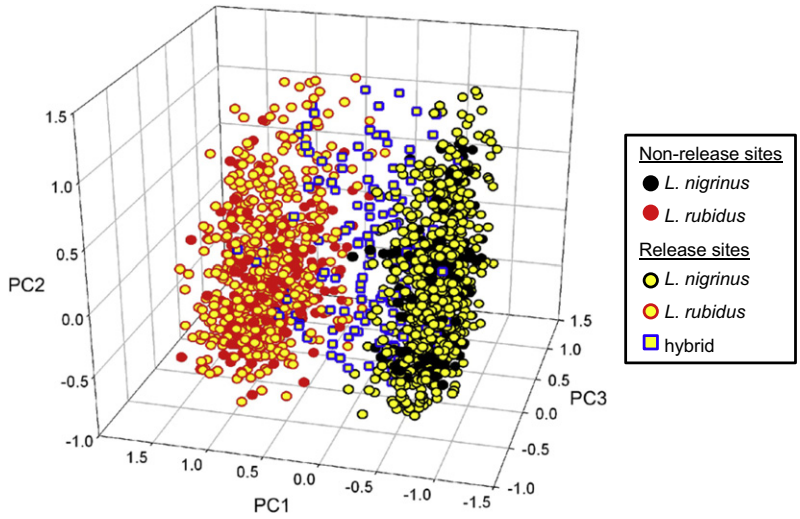

Fig. 4. Plot of the first three axes of a principal components analysis (PCA) for *Laricobius* samples collected in their native ranges and from sites where *L. nigrinus* was released as a biological control agent. The identity of individuals from release sites was determined using STRUCTURE and NEWHYBRIDS with the criteria described in Section 2.4.

elytron image. Next, with the default thresholding method, B&W threshold color, and HSB color space, the image was analyzed to calculate the black area of the elytron. Finally, the brightness threshold was increased until the entire elytron was selected and the area of the entire elytron was measured. The proportion of black was calculated by dividing the black area by the entire area.

The apices of the parameres of male genitalia are more acute in *L. nigrinus* than *L. rubidus* (Leschen, 2011; Fig. 6). To measure the angle of the parameres, digital images of slide-mounted male genitalia were acquired under a compound microscope. The images were printed and the angles of the apex of both parameres were measured using a protractor and averaged for each beetle.

The proportion of black in elytra, and the paramere angles were compared among *L. nigrinus*, *L. rubidus*, and hybrids (according to their molecular classification) with one-way ANOVA and differences among treatment means were tested with Turkey HSD ($p < 0.05$) using SPSS 14.0 (SPSS, 2005).

3. Results

3.1. Population structure within and between species

Our data included six *L. nigrinus* populations with more than 12 samples after collection sites less than 50 km apart were combined. The mean pairwise F_{ST} among all populations for this species was 0.0632, and all comparisons were significantly different from zero ($p < 0.003$ with Bonferroni correction) with the exception of Seattle, WA versus Salem, OR, and Moscow, ID versus Coeur D'Alene, ID (Supplementary Table 2). Each population had one or two loci (LaCA04 and/or LaCA14) that were not in Hardy–Weinberg equilibrium due to excess homozygotes suggesting the presence of null alleles.

When all *L. nigrinus* samples from its native range were included in STRUCTURE analysis, they clustered optimally into two groups using the ΔK method. The value Ln $P(D)$ for $K = 2$ was higher than for $K = 1$, also indicating more than one cluster (Supplementary Fig. 1). Individuals generally clustered into coastal (Oregon and Washington) versus interior sites (Idaho, Montana, and interior British Columbia) with weak but significant differentiation ($F_{ST} = 0.043$; $p < 0.0001$; Fig. 2a). Discriminant analysis of principal components (DAPC), which assigns individuals to clusters but does not rely on a model of Hardy–Weinberg equilibrium and linkage equilibrium (Jombart, 2008; Jombart et al., 2010), showed a similarly weak population structure within *L. nigrinus* (Supplementary Fig. 2).

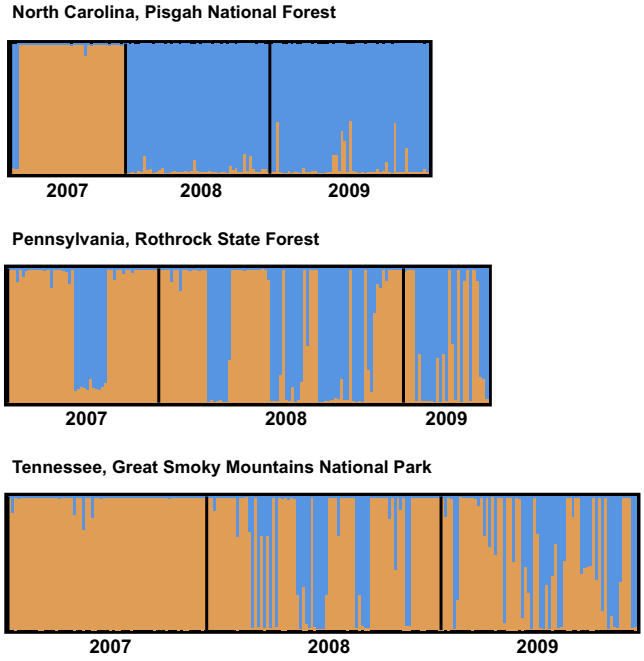

Fig. 5. STRUCTURE plots for three sites sampled over 3 years as part of a study to evaluate establishment and spread of *L. nigrinus* (Davis et al., 2012). The height of each bar represents the proportion of an individual's genotype assigned to each species. Orange corresponds to *L. rubidus* and blue to *L. nigrinus*. Vertical black lines separate individuals collected in different years. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

When all samples identified as pure *L. rubidus* from release and non-release sites were combined, there were 12 populations with more than 12 samples. Three populations had one locus (LaGT07, LaCA14, and LaCA16) that was not in Hardy–Weinberg equilibrium due to excess homozygotes suggesting null alleles. The mean pairwise F_{ST} among all populations for this species was 0.0841, and 55 out of 66 comparisons were significantly different from zero ($p < 0.0008$ with Bonferroni correction; Supplementary Table 3).

For *L. rubidus* samples from sites where *L. nigrinus* had not been released, the ΔK method returned an optimal value of $K = 3$,

however, the highest Ln $P(D)$ value resulted from $K = 1$ (Supplementary Fig. 3). There was no discernible pattern based on geography or collection year to correspond with three clusters, which suggests that $K = 1$ is optimal and that there was no population structure in this data set. With addition of samples identified as pure *L. rubidus* from release sites, the ΔK method returned an optimal value of $K = 2$ and the lowest Ln $P(D)$ value resulted from $K = 1$ (Supplementary Fig. 4), indicating two clusters in the data. Examination of the STRUCTURE plot (Fig. 2b) revealed a distinct cluster of samples from three sites in the southern Appalachians (Great Smoky Mountain National Park, TN; Dockery Lake, GA; Salt Rock Gap, NC). There was weak but significant genetic differentiation when comparing the southern Appalachian versus the remaining *L. rubidus* samples ($F_{ST} = 0.072$; $p < 0.0001$). DAPC did not detect a pattern of population structure within *L. rubidus* (Supplementary Fig. 5).

Standard Mantel tests for correlation between genetic differentiation (F_{ST}) and geographic distance was not significant for either species (*L. nigrinus*: $r = 0.1324$, $p = 0.3080$; *L. rubidus*: $r = 0.1648$, $p = 0.2320$; Supplementary Fig. 6). The partial Mantel test for the effects of coastal versus interior sites on *L. nigrinus* genetic differentiation, controlling for geographic distance, was not significant ($r = 0.2719$, $p = 0.1800$). The partial Mantel test comparing southern Appalachian *L. rubidus* versus the remaining samples was significant ($r = 0.7231$, $p < 0.001$).

There was strong differentiation between *L. nigrinus* and *L. rubidus* ($F_{ST} = 0.233$; $p < 0.0001$). The mean number of alleles per locus was 22.7 for *L. nigrinus* and 10.3 for *L. rubidus*. Expected heterozygosity (H_E) was 0.774 and observed heterozygosity (H_O) was 0.586 for *L. nigrinus*, and 0.583 (H_E) and 0.518 (H_O) for *L. rubidus*. There were deviations from Hardy–Weinberg equilibrium for all loci within *L. nigrinus*, and three loci within *L. rubidus*. The reduced observed heterozygosities may be due to a Wahlund effect caused by the presence of intraspecific population differentiation).

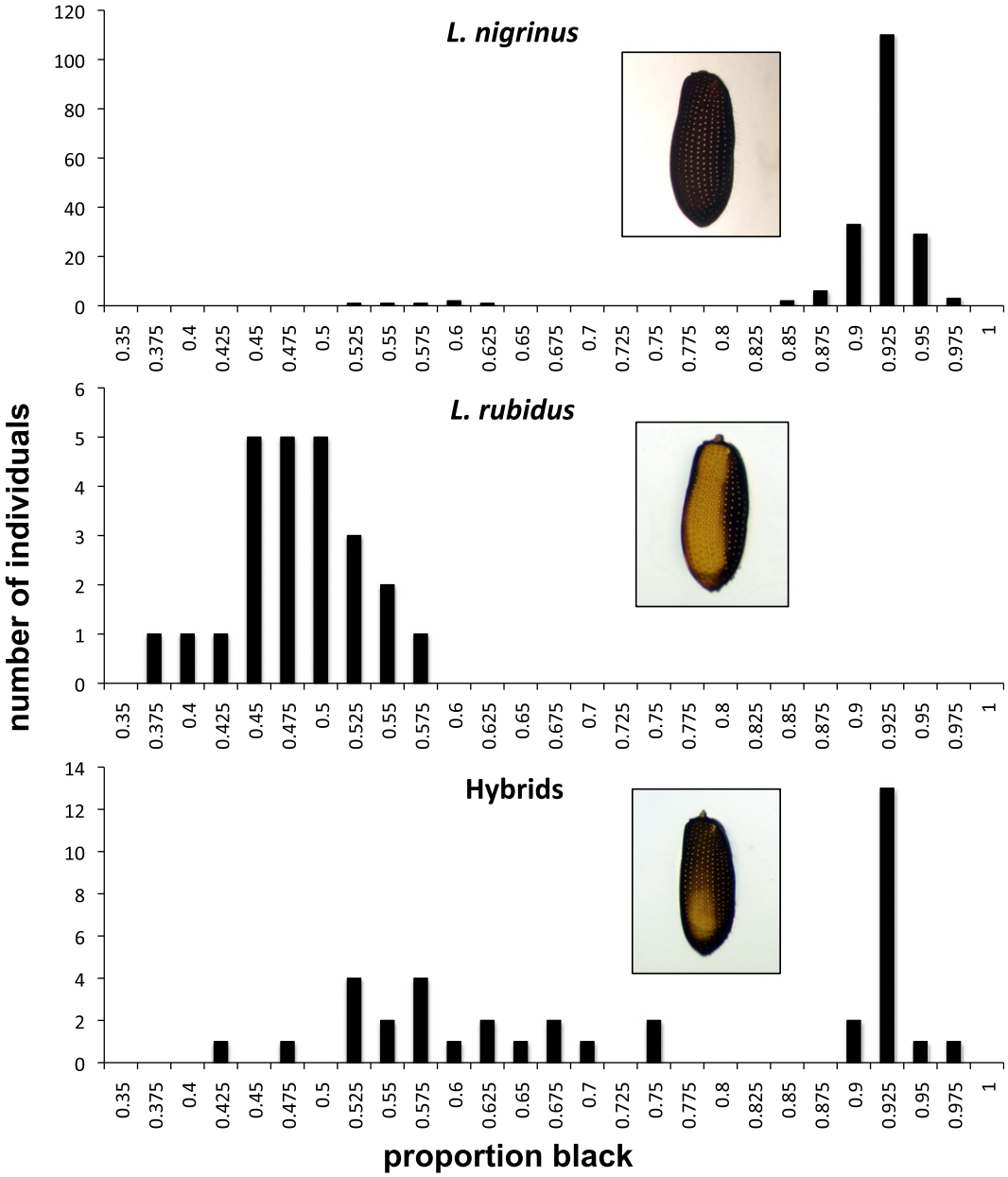

Fig. 6. Histograms of the proportion of black in beetle elytra of samples identified as *L. nigrinus*, *L. rubidus*, or hybrids. Inset photos show an elytron with the typical color pattern of each pure species and an example of an intermediate hybrid phenotype.

3.2. Evaluation of markers to distinguish hybrids from parent species

The histogram of q values resulting from Structure analysis of the simulated individuals (Fig. 3) indicated that $q = 0.80$ is a suitable cutoff value for distinguishing pure species from early generation hybrids. This cutoff value correctly identified 92% of the simulated pure *L. nigrinus*, 100% pure *L. rubidus*, 99% F1 hybrids, 92% F2 hybrids, 75% *L. nigrinus* backcrosses, and 67% *L. rubidus* backcrosses. In Newhybrids, classifying individuals to the category with the highest probability of assignment after summing hybrid classes resulted in correct assignment of 91% simulated *L. nigrinus*, 99% *L. rubidus*, 100% F1 hybrids, 94% F2 hybrids, 88% *L. nigrinus* backcrosses, and 85% *L. rubidus* backcrosses. When Structure and Newhybrids disagreed for an individual, assigning them to the category with the higher probability of the two analyses increased the overall accuracy slightly (100% for pure *L. nigrinus*, and *L. rubidus*, and F1 hybrids, 94% for F2 hybrids, 86% for *L. nigrinus* backcrosses, and 80% for *L. rubidus* backcrosses).

3.3. Estimating hybridization in L. nigrinus release sites

We generated COI sequences for 304 samples of the 1229 beetles collected from *T. canadensis* at *L. nigrinus* release sites, and RFLP scores for 901 samples. Twenty-four samples were missing COI data. Thirty-three samples were classified differently by Structure and Newhybrids. Sixteen of these were categorized as hybrids by choosing the category with the higher probability of the two analyses. Nine of the samples identified as a pure species with both Structure and Newhybrids had COI sequences matching the other species. These were classified as hybrids and are likely to be from advanced backcross generations. In total, across all sites and all collection years, there were 614 *L. nigrinus*, 475 *L. rubidus*, and 140 hybrids in release sites (Supplementary Table 1).

Of the 127 individuals classified as hybrids in Newhybrids, 76 were assigned with highest probability as F2s, and 51 as *L. nigrinus* backcrosses. None were assigned with the highest probability as F1s or *L. rubidus* backcrosses. This suggests that hybrids are fertile, and that there is a strong asymmetric introgression towards *L. nigrinus* on eastern hemlock in release sites. Of the individuals classified as hybrids, 63 had *L. rubidus* mtDNA and 75 had *L. nigrinus* mtDNA (2 hybrids were missing mtDNA data). This was not significantly different from an even ratio ($\chi^2 = 1.04$; $p = 0.307$) so there is no evidence that males or females of either species are more likely to mate with the other species.

The plot of the first three PCA axes (19.5%, 8.4%, and 6.6% of the variance respectively) showed clear separation between *L. nigrinus* and *L. rubidus* (Fig. 4). Individuals classified as hybrids were in intermediate positions but clustered asymmetrically towards *L. nigrinus*.

Fig. 5 shows Structure plots for three sites for which a standardized sampling method was applied over 3 years as part of a study to evaluate establishment and spread of *L. nigrinus* over time (Davis et al., 2012). There is a trend of increasing proportion of hybrids over time at these three sites (Pisgah: 2007 = 0.00, 2008 = 0.02, 2009 = 0.09; Rothrock: 2007 = 0.00, 2008 = 0.09, 2009 = 0.18; Smoky: 2007 = 0.03, 2008 = 0.13, 2009 = 0.29).

3.4. Morphological analyses

The proportion of black in beetle elytra was significantly different (ANOVA $p < 0.001$; Tukey HSD $p < 0.05$) among *L. nigrinus* ($N = 189$; mean = 0.90), *L. rubidus* ($N = 24$; mean = 0.47), and hybrids ($N = 38$; mean = 0.73). The histogram of the data (Fig. 6) shows a bimodal distribution for *L. nigrinus*. The larger peak (mean = 0.91) consists of entirely black individuals (the values were less than 1.0 as a result of light shining through the pores in the elytra during image acquisition). The smaller peak (mean = 0.57) consists of six individuals with red on their elytra, which is not a trait of pure *L. nigrinus*. Beetles identified as hybrids had elytra that resembled typical *L. nigrinus*, *L. rubidus*, or intermediate phenotypes. Seventeen hybrids had elytron color patterns that overlapped the typical range of *L. nigrinus* and 12 overlapped *L. rubidus*. This is not significantly different from an even ratio ($\chi^2 = 0.86$; $p = 0.650$), but is consistent with directional introgression towards *L. nigrinus*.

The angles of the parameres of the male genitalia were also significantly different (ANOVA $p < 0.001$; Tukey HSD $p < 0.05$) for all comparisons among *L. nigrinus* ($N = 50$; mean = 48.2°), *L. rubidus* ($N = 12$; mean = 72.9°), and hybrids ($N = 19$; mean = 57.4°). Eleven hybrids had paramere angles that overlapped the range of *L. nigrinus* and eight overlapped *L. rubidus* (Fig. 7). This is not significantly different from an even ratio ($\chi^2 = 0.48$; $p = 0.491$).

4. Discussion

In its native range, *L. nigrinus* exhibits weak genetic differentiation between coastal and interior populations with the intermountain region between the Cascades and the Rocky Mountains, as an incomplete barrier to gene flow. A mitochondrial COI haplotype network showed this same pattern (Davis et al., 2011). The partial Mantel test did not support this pattern, but this could be a function of low sample size, given that there were only six populations with enough samples to allow calculation of F_{ST}. The genetic differentiation between coastal and interior sites may correspond to differences in biology such as a higher cold tolerance of interior populations as shown by Mausel et al. (2011a). Specific natural enemy strains can also be associated with specific hosts (Phillips et al., 2008), which can help identify the optimal populations for biological control introduction. As of 2010, over 100,000 *L.* from coastal populations in British Columbia and Washington were released broadly in the eastern United States, and about 2600 from interior populations in Idaho and Montana were released in northeastern and mid-Atlantic states (Mausel et al., 2011b). The different populations of *L. nigrinus* could exhibit differences in prey preference or prey location behavior, as suggested by samples collected on more tree species at interior sites than at coastal sites. Systematic sampling for *L. nigrinus* on other conifer hosts of alternate adelgid prey species in both regions, and comprehensive laboratory host range testing would determine whether interior versus coastal *L. nigrinus* have different prey preferences.

L. rubidus shows weak population differentiation between samples collected in the southern Appalachians versus the rest of the sampled regions. This was evident in the Structure results as well as the partial Mantel test. Similar to the mountain ranges in the West, the Appalachian Mountains in the East are an important geographic feature that has been implicated as either an east–west barrier to gene flow or a refugium for genetic diversity during glaciation events (Jaramillo-Correa et al., 2009). Little is known about the biogeographic history of *Pinus strobus*, the preferred habitat of *L. rubidus*, except that it is closely related to *P. chiapensis* in Central America (Syring et al., 2007) with a possible Pliocene geographic connection to the latter, now separated species (Stults et al., 2010). A denser sampling of beetles in other populations and perhaps a better understanding of the biogeographic history of *Pineus strobi* on *Pinus strobus* could clarify the role that the Appalachian Mountains played in shaping *L. rubidus* genetic structure.

It is not known if the natural geographic ranges of *L. nigrinus* and *L. rubidus* are allopatric or if their distributions merge in a hybrid zone in central North America. Adelgid prey that could bridge the known distributions of these species in the boreal forests of

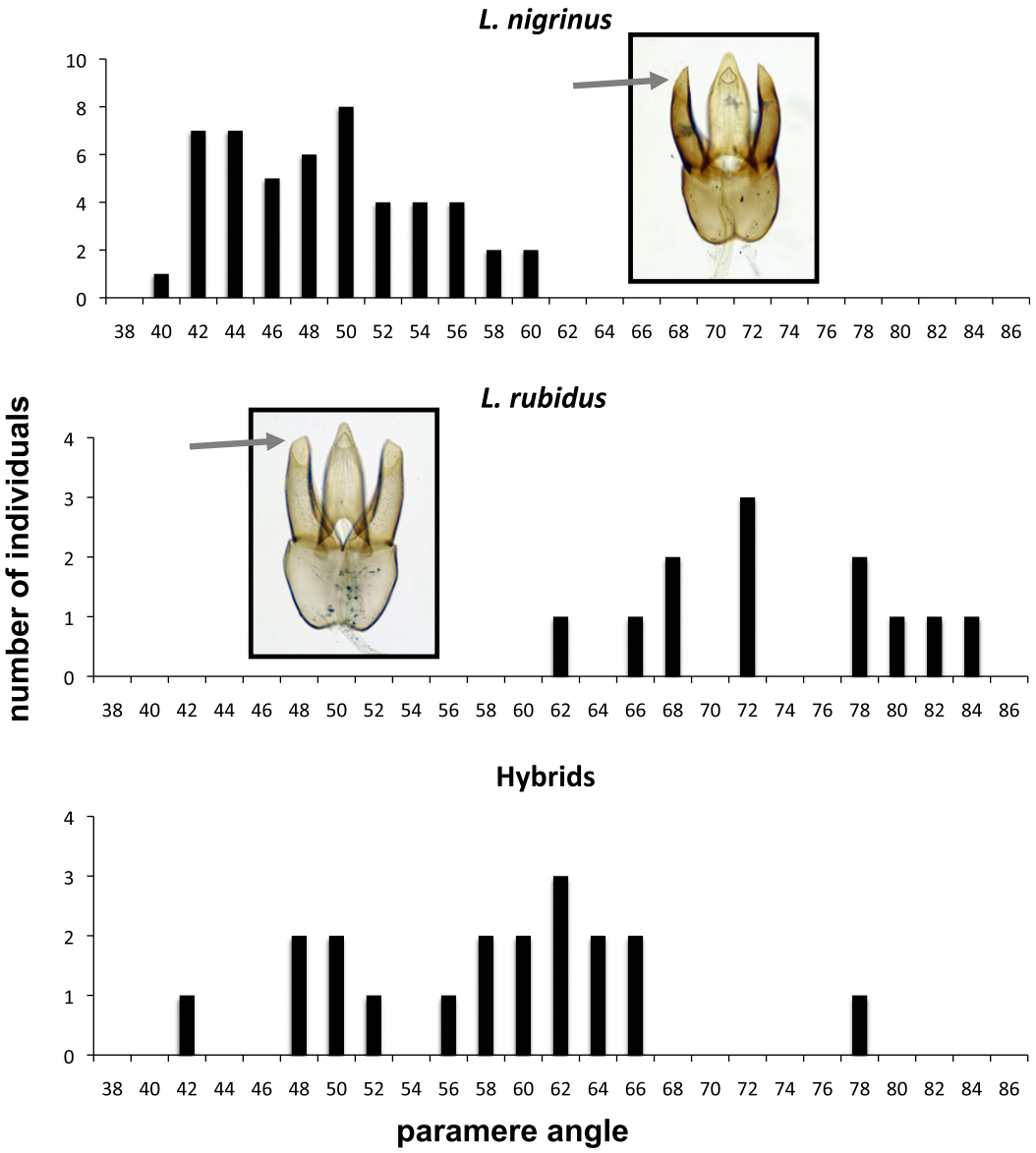

Fig. 7. Histograms of paramere angles of samples identified as *L. nigrinus*, *L. rubidus*, or hybrids using microsatellites and mitochondrial DNA haplotypes. Insets show the typical shape of male genitalia for each pure species. Parameters are indicated with arrows.

central Canada are *Adelges lariciatus* (on *Larix laricina* (Du Roi) K. Koch, *Picea glauca* (Moench) Voss, and *P. marianana* (Mill.) B.S.P), *Adelges cooleyi* (Gillette)(on *Picea glauca*), and *Pineus coloradensis* (on *Pinus banksiana* Lamb.). A collecting trip in early June 2010 to central Saskatchewan failed to locate *Laricobius* feeding on these species (N.P. Havill, unpublished). Additional collecting effort is needed in this region to determine how far the geographic range of each species extends. Knowing whether these species diverged in isolation, or with gene flow while adapting to different prey would help to predict the outcome of the newly emerging hybrid zone in the eastern United States. Reproductive incompatibility generally increases with divergence time (Coyne and Orr, 1997; Edmands, 2002), however, we might expect reinforcement of reproductive isolation to be more likely during secondary contact if this were a mechanism that helped drive their initial divergence.

The two most common methods for assessing hybridization are implemented by Structure and Newhybrids. Both methods are efficient in detecting early generation hybrids, but are sensitive to the number of loci and the genetic distance between parent groups (Vähä and Primmer, 2006; Sanz et al., 2009). Our simulations show that given the strong genetic differentiation between *L. nigrinus* and *L. rubidus* (F_{ST} = 0.233), six microsatellite markers with a cutoff of q = 0.80 is adequate for distinguishing pure species from early hybrid classes. However, many later generation backcross hybrids are likely to be undetected resulting in a conservative estimate of the rate of hybridization. Indeed, the six individuals classified as *L. nigrinus* that had red on their elytra (Fig. 6) are probably later generation hybrids that were not detected using molecular methods. Inclusion of mitochondrial haplotypes identified additional advanced hybrids, but considering the additional cost of screening this locus, it may not be necessary for simply detecting hybrids in a population or comparing relative rates of introgression over time. The morphological characters used to distinguish the species (elytron color and male paramere angle) are not reliable characters for identifying hybrid individuals because the variation among hybrids broadly overlaps both parent species (Figs. 6 and 7).

Hybridization between *L. nigrinus* and *L. rubidus* is in its early stages, but we can make some tentative predictions about its outcome pending additional studies. A particular hybridization event can range from very little genetic introgression when

matings are rare or hybrids have low fitness, to complete admixture when the homogenizing effects of gene flow overwhelm any existing isolating mechanisms (Allendorf et al., 2001). Hybridization between *L. nigrinus* and *L. rubidus* does not appear to be at the lower end of this spectrum. There is strong evidence for hybrid fertility and advanced generation hybrids, which shows that hybridization is broadly occurring. However, the persistence of individuals identified as pure species and asymmetrical introgression suggest that complete admixture may not occur.

The relative fitness of hybrids versus the parent *Laricobius* species would also help to predict the outcome of hybridization. There could be a negative effect on biological control if hybrids feed less on the target pest (Goldson et al., 2003), or interbreeding results in wasted reproductive effort (Stouthamer et al., 2000). Reduced fitness of hybrids could result in a hybrid zone with a steep genetic cline between species where their preferred habitats meet (Hewitt, 1988). Since the preferred habitats of each *Laricobius* species (hemlock and white pine) broadly overlap in eastern North America, this could take the form of a mosaic hybrid zone (Harrison and Rand, 1989) with variable patches of interbreeding that depend on habitat distribution and the rates of release and dispersal of *L. nigrinus*.

There is good evidence for reproductive isolation between closely related herbivorous insects based on host plant preference (Matsubayashi et al., 2010), but not for specialist predators that use plant cues to find their prey. In laboratory tests, Wallin et al. (2011) found that *L. nigrinus* was attracted to volatiles from eastern hemlock (*T. canadensis*), western hemlock (*T. heterophylla*), western white pine (*Pinus monticola*), and white spruce (*Picea glauca*), but not to hemlock adelgids alone, showing that cues from adelgid host plants are important for prey location. The strong asymmetric introgression towards *L. nigrinus* on eastern hemlock suggests that the western species has more of an affinity for this habitat than the native *L. rubidus*. Alleles that improve location of one prey species may not be advantageous in other habitats and selection for these alleles could ultimately reinforce isolation between the predatory species. More work is needed to test the prey preferences and prey location behavior of each species and their hybrids to assess whether these traits will promote reproductive isolation between the species. In addition, since this study focused on sampling eastern hemlock, collecting beetles from white pine and determining whether there is a complementary pattern of asymmetrical introgression in the preferred habitat of *L. rubidus* will further help to predict the outcome of hybridization. Introgression of genes between species could also change their host preferences over time, so this should be carefully monitored as hybridization progresses.

Hybridization could improve establishment of *L. nigrinus* and accelerate its adaptation to the new and unoccupied niche of *A. tsugae* on eastern hemlocks. The ability to hybridize with *L. rubidus* could promote establishment by alleviating Alee effects associated with finding mates. In addition, hybridization, even at very low frequency, can be a source of new allelic combinations that can offset inbreeding depression and the loss of genetic variation due to drift, regardless of whether hybrids are more or less fit than the parents (Lewontin and Birch, 1966; Gompert et al., 2006; Grant and Grant, 2010). For example, introgression of genes from *L. rubidus* could improve the ability of hybrids to survive in the eastern climate relative to pure *L. nigrinus*.

This study confirms the importance of determining whether biological control agents can hybridize with closely related native taxa, and when found, evaluating the impact on biological control and native biodiversity. Future work with *L. nigrinus* and *L. rubidus* should evaluate the fitness of each species relative to hybrids in the lab and in the field in different habitats and with different prey. This will help determine the effects on biological control and on the integrity of native *L. rubidus*. This study system also provides an excellent opportunity to improve our understanding of emerging hybrid zones by tracking its progress over time.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2012.08.001.

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