



Field assessment of hybridization between *Laricobius nigrinus* and *L. rubidus*, predators of Adelgidae



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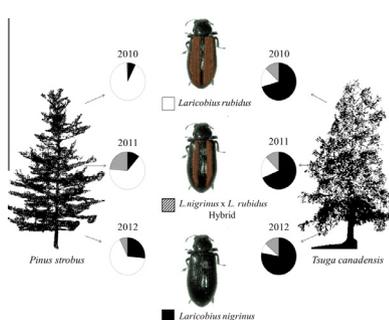
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HIGHLIGHTS

- We examined population changes among two *Laricobius* species and their hybrids.
- On hemlock, *L. nigrinus* populations increased and *L. rubidus* populations decreased.
- *Laricobius nigrinus* was dominant on hemlock; *L. rubidus* was dominant on white pine.
- Hybrids were dominant on hemlock and their populations remained steady over time.
- Stable hybrid zones will most likely be maintained.

GRAPHICAL ABSTRACT



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ABSTRACT

Two adelgid predators, *Laricobius nigrinus* Fender and *Laricobius rubidus* LeConte, were recently discovered to produce hybrid progeny in the eastern United States. *L. rubidus* is native to eastern North America where it feeds on pine bark adelgid (*Pineus strobi* Hartig) and *L. nigrinus* is native to western North America and was introduced to the eastern United States in 2003 for biological control of hemlock woolly adelgid (*Adelges tsugae* Annand). Currently, *L. nigrinus* and *L. rubidus* form a mosaic of hybrid zones throughout the eastern United States. It is not known whether these zones will be maintained over time and whether hybridization will impact the efficacy of biological control or result in displacement of *L. rubidus*. Sampling from 2007 to 2012 on eastern hemlock (*Tsuga canadensis* Carrière) showed a clear increase in *L. nigrinus*, a decrease in *L. rubidus*, and a steady proportion of hybrids. Sampling from 2010 to 2012 on both eastern hemlock and white pine (*Pinus strobus* L.) at ten sites showed that *L. nigrinus* was more dominant on hemlock and *L. rubidus* was more dominant on white pine, which may demonstrate habitat preference and promote segregation between the two species. Site factors were tested for a relationship with the proportion of hybrids. The number of years *L. nigrinus* had been present at the site was the only factor that showed a relationship. The results suggest that *L. nigrinus* may displace *L. rubidus* on hemlock, but not on white pine at sites where they are both present.

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1. Introduction

Laricobius nigrinus Fender and *Laricobius rubidus* LeConte are predatory beetles in the family Derodontidae that feed exclusively

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on adelgids (Hemiptera: Adelgidae). *Laricobius rubidus* is native to eastern North America and feeds primarily on pine bark adelgid, *Pineus strobi* Hartig (hereafter PBA) (Clark and Brown, 1960), while *L. nigrinus* is native to western North America where it feeds primarily on hemlock woolly adelgid, *Adelges tsugae* Annand (hereafter HWA) (Havill et al., 2012; Mausel et al., 2011; Zilahi-Balogh et al., 2002). *L. nigrinus* and *L. rubidus* are sister species that recently diverged from a common ancestor (Davis et al., 2011; Montgomery et al., 2011), although the drivers of this divergence are unknown. Beginning in 2003, *L. nigrinus* was released in the eastern United States as a biological control agent of HWA. Several years after releases began, it was discovered that *L. nigrinus* was hybridizing with *L. rubidus* (Havill et al., 2012).

Currently, *L. nigrinus* and *L. rubidus* form a mosaic of hybrid zones in areas where *L. nigrinus* was introduced in the eastern United States (Havill et al., 2012). A mosaic pattern probably formed because: (1) *L. nigrinus* has been released across the landscape in a patchy pattern; (2) the host plants of the preferred prey of each *Laricobius* species [hemlocks, *Tsuga canadensis* (L.) Carrière and *Tsuga caroliniana* Engelmann, and white pine, *Pinus strobus* (L.)] broadly overlap in the region where the beetles are hybridizing; and (3) *L. rubidus* can still be found in large regions of allopatry (Havill et al., 2012), particularly where white pine and hemlock are not in close proximity or in mixed stands where *L. nigrinus* has yet to become established. It is not known whether the two *Laricobius* spp. will maintain these hybrid zones in a stable form that maintains the genetic distinction between the species. Additionally, each individual patch within the mosaic hybrid zone could have a unique evolutionary trajectory resulting in several different outcomes depending on different site factors (Harrison and Rand, 1989). Potential outcomes include genetic assimilation or displacement of *L. rubidus* or *L. nigrinus*, reinforcement of premating isolation resulting in decreased hybridization and permanent separation of *L. nigrinus* and *L. rubidus*, creation of a third species, or maintenance of stable hybrid zones.

Any of these outcomes could impact the efficacy of biological control of HWA using *L. nigrinus*. For this reason, and because very little is known in general about the extent and impact of hybridization between introduced biological control agents and native species (Hopper et al., 2006), there is a need to monitor the outcome of this particular interaction. Therefore, a study was initiated in 2010 to better understand the interactions of *L. nigrinus* and *L. rubidus* in hybrid zones. The objectives of this study were to use genetic markers to: (1) determine the status of hybridization at ten study sites where *L. nigrinus* has been released; (2) document how hybridization is changing over time; (3) infer prey preference of hybrids in a natural setting; and (4) identify site factors that may drive hybridization. This information may help predict the evolutionary trajectory of the interaction between *L. nigrinus* and *L. rubidus*.

2. Methods

Ten sites where *L. nigrinus* had been previously released were chosen for this study (Table 1). Site attributes including latitude, elevation, the number of *L. nigrinus* released and the year of release were obtained from Mausel (2007) and the HWA Predator Release and Monitoring Database (hwa.ento.vt.edu/hwa/hwa.cgi). *L. rubidus* was known to be present at all sites except Burns Creek and Devil's Fork (Davis et al., 2012). Hybridization was already known to have been occurring at three of the sites (Laurel Creek, Middle Creek, and Rothrock) (Havill et al., 2012). All sites were visited once per year in the spring (2010–2012), when *Laricobius* larvae were most numerous.

At each site, 16 hemlock and 16 white pine points were chosen based on the presence of an adelgid infestation. Approximately 25 adelgid-infested branches (30 cm long) were collected from each point, which included the center tree as well as infested trees within a 3.6 m radius from the plot center. These branches were placed in plastic bags that were filled with air and sealed to keep from crushing any larvae present during transport. The hemlock and white pine branches were brought back to the Virginia Tech Insectary and placed in funnels to rear larvae to the prepupal stage (Salom et al., 2012). Branches from two different points were placed in one funnel, thereby partially grouping the samples by tree species, resulting in a total of eight hemlock funnels and eight white pine funnels per site. For each funnel, all prepupal larvae were collected and placed in vials with 95–100% ethanol. Ten larvae were randomly chosen from each funnel for genetic analysis each year.

Basal area per hectare (BA/Ha) and trees per hectare (TPH) measurements were collected at each site to determine if density of eastern hemlock and/or eastern white pine had an influence on the proportion of *Laricobius* species and hybrids found at each site. Basal area per hectare and TPH was measured using 40 m² fixed area plots (Avery and Burkhart, 2002). All trees with a diameter at breast height (1.37 m above ground) ≥ 2.54 cm were included in the BA/Ha measurements and all trees were included in the TPH measurements. Trees per hectare were measured in addition to BA/Ha because a large proportion of PBA-infested branches were collected from seedling (<5.08 cm) or sapling (between 5.08 and 10.16 cm) size white pine trees. At some sites, the incidence of these smaller white pine trees was significant. By collecting this additional measurement, the smaller trees and therefore, the potential presence of *L. rubidus* larvae, could be accounted for during the study.

2.1. Genetic analysis

Larvae used for genetic analysis were dissected; the heads were collected and placed as voucher specimens in the Virginia Tech

Table 1
The latitude, elevation, percent hemlock basal area per hectare (BA/Ha) and trees per hectare (TPH), percent white pine BA/Ha and TPH, year of first *Laricobius nigrinus* release at each site (release year), and the total number of *L. nigrinus* (Ln) released at each site as of 2012.

State	Forest/park	Site	Latitude	Elevation	% Hemlock		% White pine		Release year	No. of Ln released
					BA/Ha	TPH	BA/Ha	TPH		
TN	Great Smoky Mtn. Natl. Park	Laurel Creek	35.60353	630.48	3.70	2.94	1.08	4.16	2004	300
NC	Pisgah Natl. Forest	Middle Creek	35.79400	940.11	2.81	9.28	0.00	0.10	2005	600
VA	Jefferson Natl. Forest	Burns Creek	36.92606	646.68	36.71	19.89	0.00	0.00	2008	300
VA	Jefferson Natl. Forest	Devil's Fork	36.81988	514.83	39.05	10.08	0.00	0.00	2008	300
MD	Rocky Gap State Park	Rocky Gap	39.28085	234.41	16.59	8.25	2.37	1.87	2004	3476
VA	Jefferson Natl. Forest	North Fork	37.44344	792.42	15.06	9.71	6.23	9.50	2003	600
PA	Bald Eagle State Forest	Bear Run	40.89871	394.10	6.29	11.89	5.21	11.42	2005	300
PA	Bald Eagle State Forest	Poe Valley	40.82619	416.77	24.14	51.39	0.25	4.64	2008	300
PA	Rothrock State Forest	Rothrock	40.65508	390.46	27.21	26.01	1.00	11.92	2003	897
PA	Rothrock State Forest	Treatser Valley	40.79569	460.46	23.46	27.90	6.66	9.41	2007	300

Insect Museum. The remainder of the specimen was used to extract DNA using the DNeasy kit (Qiagen Inc., Valencia, CA). The identities of *Laricobius* larvae were determined using six nuclear microsatellite loci (LaGT01, LaCA04, LaGT07, LaGT13, LaCA14, LaCA16) (Havill et al., 2012; Klein et al., 2010) which were amplified using the conditions described in Klein et al. (2010). Fragment analysis was completed at the DNA Analysis Facility on Science Hill at Yale University in New Haven, CT using a 3730xl 96-capillary genetic analyzer (Applied Biosystems). Genotypes were scored using Genemapper 4.0 (Applied Biosystems). The programs Structure 2.3.2 (Pritchard et al., 2000) and NewHybrids 1.1 (Anderson and Thompson, 2002) were used to distinguish hybrids from parent species using the criteria described in Havill et al. (2012). Briefly, Structure runs used 20,000 burn-in iterations followed by 100,000 sample iterations and NewHybrid runs were completed with 10,000 burn-in iterations followed by 100,000 sample iterations. For NewHybrids, the probabilities of assignment to all four potential hybrid classes (F_1 , F_2 , *L. rubidus* backcrosses, and *L. nigrinus* backcrosses) were summed to obtain the probability of assignment as a hybrid. The results of five independent runs were averaged for Structure and NewHybrids. All runs included samples of pure *L. nigrinus* ($n = 198$) and *L. rubidus* ($n = 161$) that were coded as having known ancestry in both Structure and NewHybrids (see Havill et al. 2012 for collection information of known specimens). Each larva was assigned to a parental species if Structure analysis resulted in $q > 0.80$, or as a hybrid if $0.20 < q < 0.80$. If the category with the highest probability of assignment from NewHybrids agreed with Structure, that assignment was retained. If they did not agree, the beetle was assigned to the category with the higher probability of the two analyses. Simulations show that this method is highly accurate for distinguishing the two *Laricobius* species and their early generation hybrids (Havill et al., 2012).

2.2. Statistical analysis

For the following analyses, JMP Pro 10 and an alpha of 0.05 were used when relevant. Data from the Laurel Creek, Middle Creek, and Rothrock site were available from a previous study that took place from 2007 to 2009 (Davis et al., 2012), which used the same methodology to collect *Laricobius* larvae, but from hemlock only. These data were added to the data collected from hemlock at these sites from 2010 to 2012 to analyze changes in hybridization and parental species composition using linear regression analysis.

A contingency analysis followed by a Cochran–Mantel–Haenszel test (Conover, 1999, SAS Institute Inc., 2012) with year as a blocking factor (to account for the effect of year) was used to determine whether the distributions of *L. nigrinus*, *L. rubidus*, and hybrids on hemlock and white pine across years (2010–2012) were similar (Ott and Longnecker, 2010). The total number of *L. nigrinus*, *L. rubidus*, and hybrids on white pine and hemlock at all 10 sites for 2010–2012 was used for this analysis.

Several factors or variables were recorded to determine if specific characteristics of the sites where *L. nigrinus* was released had an effect on the proportion of hybrids. These variables included: latitude, elevation, hemlock and white pine BA/Ha, hemlock and white pine TPH, the number of *L. nigrinus* released at each site, and the number of years *L. nigrinus* was present at the site.

A principal components analysis (PCA) (Dunteman, 1989) was performed to test for multicollinearity among the eight site variables and to identify a subset of representative variables for regression analysis (Dunteman, 1989, Hair et al., 2010). The PCA was carried out on the correlation matrix and four principal components were selected for further analysis based on the scree plot (Dunteman, 1989). The variables with the largest loadings on each of the four selected principal components were used in a stepwise

regression analysis to determine which one or group had the greatest influence on the percent of hybrids found at the sites.

3. Results

By 2011, hybridization was observed at all sites except at Devil's Fork. Neither pure *L. rubidus* larvae nor hybrids were found at this site. This is the only site in the study where white pine was not present.

3.1. *Laricobius* populations

At the three sites where *Laricobius* population data were available from 2007 to 2012 (i.e., Laurel Creek, Middle Creek, and Rothrock), the proportion of *L. nigrinus* increased progressively over time, while *L. rubidus* decreased; a relatively steady proportion of hybrids was also observed after their initial appearance in 2008 (Fig. 1). Linear regression showed that the increase in the proportion of *L. nigrinus* over time was significant ($F = 22.840$, $df = 1$, $P = 0.009$, $R^2 = 0.85$), as was the decrease in the proportion of *L. rubidus* on hemlock ($F = 15.644$, $df = 1$, $P = 0.0167$, $R^2 = 0.80$).

3.2. Hemlock vs. white pine

L. nigrinus was found more often on hemlock than white pine in all three years (Fig. 2). *L. rubidus* was found on both hemlock and white pine, but was always more abundant on white pine than *L. nigrinus*. The hybrids were found on both tree species in 2011, but almost solely on hemlock in 2010 and 2012 (Fig. 2).

Contingency analysis showed that there was a significant difference in the distributions of the *Laricobius* species on hemlock and white pine across years, 2010–2012 ($\chi^2 = 550.306$, $df = 2$, $P < 0.0001$). A significant difference was also observed in the distributions of *Laricobius* species on hemlock and white pine ($\chi^2 = 504.184$, $df = 2$, $P < 0.0001$) after adjusting for the effects of year with the Cochran–Mantel–Haenszel test. Within species distributions with respect to the two tree species showed that percent *L. nigrinus*, *L. rubidus*, and hybrids were greater (95.69), similar (43.72), and greater (76.10) on hemlock, respectively compared with white pine. Within tree distributions show that the proportion of *L. nigrinus* was greatest on hemlock compared with the other two beetle types (Ln: 72.4%, Lr: 14.7%, Hybrid: 12.97%). The

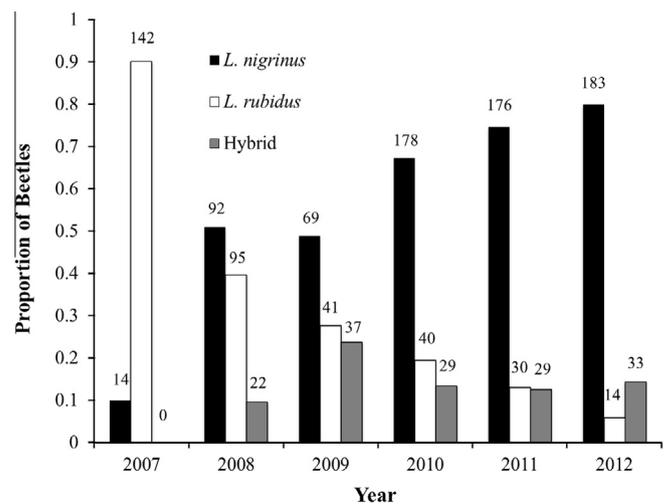


Fig. 1. The average proportion of *Laricobius nigrinus*, *L. rubidus*, and their hybrids found on hemlock at the Laurel Creek, Middle Creek, and Rothrock sites from 2007 to 2012, with the absolute number displayed above each bar.

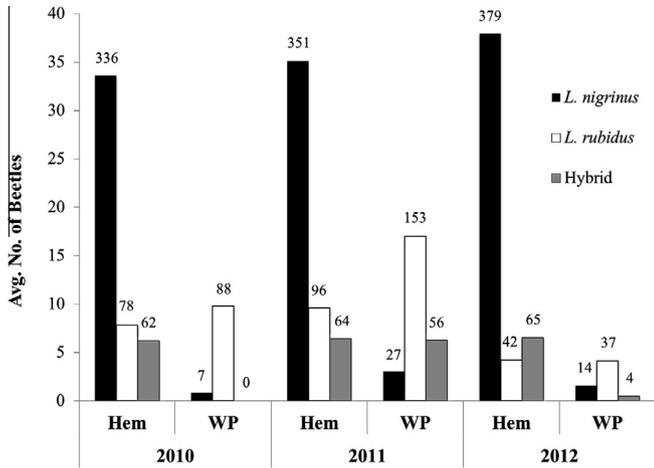


Fig. 2. The average number of *Laricobius nigrinus*, *L. rubidus*, and their hybrids found on eastern hemlock and white pine in 2010, 2011, and 2012 with the absolute number displayed above each bar.

proportion of *L. rubidus* was greatest on white pine (Ln: 12.4%, Lr: 72.02%, Hybrid: 15.5%).

All hybrids found from 2010 to 2012 were identified as F₂'s using the program NewHybrids, which suggested that F₁ hybrids are not sterile; however, with only 6 loci, the ability of assigning a particular hybrid class is limited. On hemlock, there was a clear pattern of asymmetrical introgression towards *L. nigrinus* (Fig. 3). Of the *Laricobius* found on white pine, there was a less discernable pattern of asymmetrical introgression towards *L. rubidus*, but there were a high proportion of pure *L. rubidus* individuals (Fig. 4).

3.3. Site factors affecting hybridization

The four variables selected for stepwise regression following PCA were hemlock TPH, white pine BA/Ha, the number of years *L. nigrinus* was present at the site, and the number of *L. nigrinus* released at each site. Mixed regression analysis showed that the number of years that *L. nigrinus* was present at the site was the only factor related to percent of hybrids (Fig. 5; $P=0.004$, $R^2=0.30$). The other three factors (hemlock TPH, white pine BA/Ha, and the number of *L. nigrinus* released at the sites) did not significantly affect the percent hybrids found at these sites (Table 2).

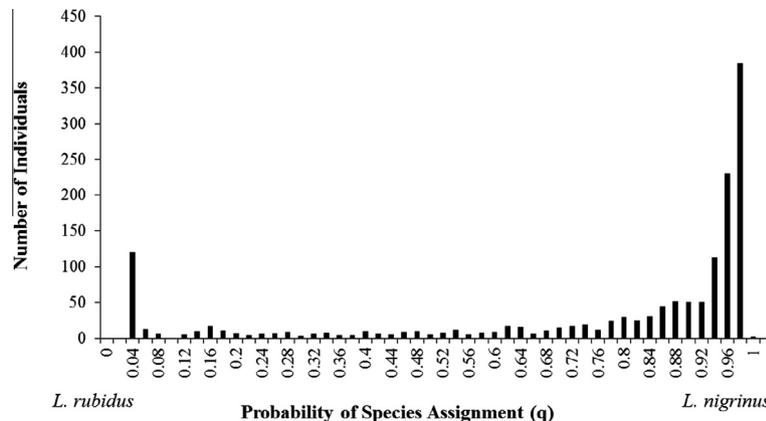


Fig. 3. Histogram of the probability of species assignment (q) resulting from analysis of *Laricobius* on eastern hemlock. A value of zero represents pure *L. rubidus*; a value of one represents pure *L. nigrinus*; 0.50 represents a hybrid with equal contribution from each parent species.

4. Discussion

4.1. Potential outcomes of hybridization

4.1.1. Genetic assimilation/displacement

Genetic assimilation is the loss of a species as genetic introgression occurs from a related species causing one to become more like the other. The complete loss of either *Laricobius* species through genetic assimilation may not occur because populations of the two species vary from site to site (e.g. number of *L. nigrinus* released), local selection pressures vary (e.g. percent of hemlock and/or white pine present), each species appears to have different habitat preferences, and *L. rubidus* populations exist outside the eastern populations of *L. nigrinus*. Factors such as these may promote the persistence of each distinct species in spite of gene flow, reducing the probability of assimilation and extinction (Harrison and Rand, 1989).

Introgression could result in hybrids that have no host preference, or preference for HWA or PBA. This study showed hybrids were proportionally more abundant on HWA (76.10) than PBA. This could influence populations of PBA on eastern white pine, but would likely require that *L. rubidus* lose its genetic integrity (which is contrary to the observations in this study; Fig. 4), and require *L. rubidus* to be a more important predator than other specialist natural enemies, such as silver flies (Chamaemyiidae), in the maintenance of PBA populations. If in the future, hybrids prefer PBA to HWA; this could result in a decrease in the efficacy of *Laricobius* as a biological control agent and/or affect competition with other native predators of PBA.

The increase in the number of pure *L. nigrinus* individuals over time suggests that this species is not becoming assimilated or being displaced through hybridization. Data from 2007 to 2012 show a clear decrease in *L. rubidus*, but this is occurring only on hemlock (Fig. 1). It is unclear whether *L. rubidus* are also decreasing on white pine (Fig. 2). If they are, it is most likely not due to competition with *L. nigrinus* for PBA, since *L. nigrinus* is found in very low numbers on white pine (Fig. 2).

4.1.2. Reinforcement of premating isolation

Reinforcement is an increase in premating isolation between species in response to any type of selection against hybridization, including habitat preference, and temporal, behavioral, mechanical, and/or gametic differences (Servedio and Noor, 2003). Reinforcement might be a likely expectation if this were a mechanism that helped drive the initial divergence. In the case of

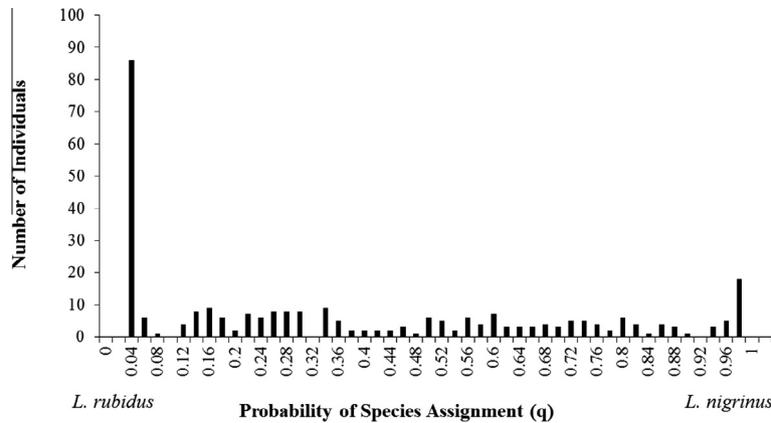


Fig. 4. Histogram of the probability of species assignment (q) resulting from analysis of *Laricobius* collected on eastern white pine. A value of zero represents pure *L. rubidus*; a value of one represents pure *L. nigrinus*; 0.50 represents a hybrid with equal contribution from each parent species.

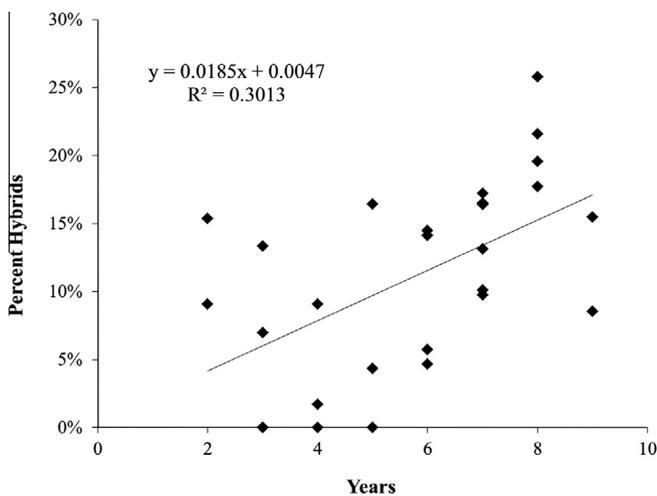


Fig. 5. Relationship between percent hybrids and the number of years *Laricobius nigrinus* had been present at 10 sites from 2010 to 2012.

Table 2

Results of a mixed regression analysis examining the relationship between site factors (number of years *L. nigrinus* was present at site, hemlock trees per hectare (TPH), white pine basal area per hectare (BA/Ha) and the number of *L. nigrinus* released) and the percent of *Laricobius nigrinus* \times *L. rubidus* hybrids collected at 10 sites from 2010 to 2012.

Site factors	df	F ratio	Prob > F
No. of years <i>L. nigrinus</i> was present at site	1	10.025	0.0039
Hemlock TPH	1	0.069	0.7951
White pine BA/Ha	1	0.089	0.7676
No. of <i>L. nigrinus</i> released	1	0.012	0.9148

L. nigrinus and *L. rubidus* it is not clear what led to their divergence. Some possibilities are: (1) allopatric divergence due to historical changes in the distributions of the conifer hosts of their adelgid prey; (2) initial sympatric divergence associated with specialization on different adelgid prey (for example on hemlock adelgids vs. pine adelgids in western North America) followed by allopatric divergence; or (3) isolation by distance, if they are still found in sympatry somewhere in central North America where adelgid prey such as larch adelgids in central Canada bridge the known distribution of the two species. If *L. nigrinus* and *L. rubidus* did diverge in allopatry, adaptations that increase reproductive isolation would have been unnecessary and therefore would be one of the last features to differentiate (Hoskin et al., 2005). Given that *L. nigrinus*

and *L. rubidus* can successfully mate, this scenario is a possibility. It is also possible that the two species meet somewhere between their current known distributions, forming a natural hybrid zone where gene flow between the two species is presently occurring. There is no evidence of this to date, but there has been very limited sampling in the region where natural hybridization would be most likely to occur (Havill et al., 2012).

If reinforcement is occurring between the two *Laricobius* species, over time we will see a decrease in hybridization and eventually a termination of hybridization as reinforcement leads to permanent speciation. In this study, percent hybrids remained relatively stable; therefore we do not have evidence at this point that reinforcement is occurring.

4.1.3. Hybrid vigor

When HWA was introduced into the eastern U.S. from Japan, a new niche was created for specialist predators. *Laricobius* hybrids could be better adapted to this niche than their parents if they receive a preference for HWA from their *L. nigrinus* parents, and hardiness in eastern climates from *L. rubidus* (Havill et al., 2012). Hybridization was relatively stable through the three years of this study and an increasing number of individuals on hemlock were identified as pure *L. nigrinus*, which may suggest that the hybrids are not better adapted than their parents in this niche.

Hybridization could accelerate adaptation of *L. nigrinus* to HWA on eastern hemlock through introgression. The increase in *L. nigrinus* populations over time could be due to accelerated adaptation as a result of introgression of favorable genes, or simply a numerical response to the abundance of food and favorable habitat.

4.1.4. Speciation

Hybrids tend to be best adapted to conditions intermediate to those of the parental optima (Barton, 2001). Extreme habitats relative to the requirements of parents, rather than habitats with characteristics intermediate between the parental niches are typically necessary for speciation to occur (Buerkle et al., 2000). In the case of *L. nigrinus*, *L. rubidus*, and their hybrids, there is no evident extreme habitat. Additionally, because divergence between the two species is low, there may be little chance for major novelties to arise in their hybrids (Abbott et al., 2013). Given these factors, it is unlikely that *L. nigrinus* \times *L. rubidus* hybrids will form a new species distinct from their parent species.

4.2. Conclusion

Based on the data presented here, two possible outcomes of the interaction between *L. nigrinus* and *L. rubidus* may be: (1) *L. nigrinus*

and *L. rubidus* will maintain stable hybrid zones with steady hybridization between the two species; and/or (2) *L. nigrinus* may outcompete *L. rubidus* on hemlock at sites where they are both present. These scenarios seem likely because on hemlock there has been an increase in *L. nigrinus* populations, a decrease in *L. rubidus* populations, and steady levels of hybridization, while the data from both hemlock and white pine do not show an overall decrease in *L. rubidus* populations.

It is clear from the analysis of *Laricobius* distribution on hemlock vs. white pine (Fig. 2) and from the histograms showing species assignment for *Laricobius* collected on hemlock and white pine (Figs. 3 and 4) that *L. nigrinus* prefers hemlock while *L. rubidus* prefers white pine. This suggests that isolation between the two species due to habitat preference may occur, but stable hybrid zones may still persist if occasional gene flow continues between the species.

The only site factor that had any relationship with percent of hybrids at these sites was the number of years *L. nigrinus* was present at the site. This asserts that hybridization can occur after release of *L. nigrinus*, and it can occur at an increasing rate as *L. nigrinus* becomes more abundant and contacts between *L. nigrinus* and *L. rubidus* become more frequent, perhaps to a plateau as *L. nigrinus* outcompetes *L. rubidus* on hemlock and contact between the two species stabilizes.

While the evidence collected here may point to the potential outcomes of hybridization listed above, there may be several other factors affecting the results of this study: (1) the analysis used to determine the identities of the *Laricobius* spp. is coarse, and it is possible that later generation backcross hybrids were not detected and some beetles identified as pure parental species were in fact hybrids (Havill et al., 2012); (2) changes in weather conditions (e.g., precipitation and temperature) from year to year during collection could have had an effect on the number of *L. nigrinus*, *L. rubidus*, and hybrids collected. This would depend upon the climatic preference of each species; an attribute that is not well known in relation to *L. rubidus* or the hybrids; (3) the proportions of beetle species could be affected by changes in the abundance of their prey from year to year; and (4) it is likely that not enough time has passed since the two *Laricobius* spp. were placed back into contact to determine the final evolutionary outcome.

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