2014 *Southeastern Naturalist* 13(Special Issue 6):178–191

Contamination Delays the Release of *Laricobius osakensis* for Biological Control of Hemlock Woolly Adelgid: Cryptic Diversity in Japanese *Laricobius* spp. and Colony-Purification Techniques

Melissa J. Fischer[1,*], Nathan P. Havill[2], Carrie S. Jubb[1], Sean W. Prosser[3], Brent D. Opell[4], Scott M. Salom[1], and Loke T. Kok[1]

Abstract - *Laricobius osakensis* (Coleoptera: Derodontidae) was imported from Japan to the United States in 2006 for study in quarantine facilities as a potential biological control of Hemlock Woolly Adelgid. *Laricobius osakensis* was released from quarantine in 2010, but it was soon discovered that the colony also contained a cryptic species, *Laricobius naganoensis*. This led to the placement of *L. osakensis* back into quarantine and development of a method to purify the colony. To distinguish the two species, we designed a restriction fragment length polymorphism (RFLP) assay using mitochondrial DNA sequences and developed a non-lethal testing method. Twenty-one diagnostic nucleotide sites separated the two species, and they both exhibited extraordinary intra-specific haplotype diversity. Sequencing the ITS2 nuclear region did not produce evidence of hybridization between the species in the field or in the lab colony. Splitting the colony into small groups and testing their species composition was successful in isolating *L. osakensis.* Efforts should be made to maintain high genetic diversity in *L. osakensis* colonies. Continued genotyping of new colony stock will be necessary to fully characterize the diversity within both species.

Introduction

Laricobius osakensis Shiyake and Montgomery was imported into the US under quarantine from Japan in 2006 for study as a potential biological control agent of the invasive *Adelges tsugae* Annand (Hemlock Woolly Adelgid). *Laricobius osakensis* was collected in Japan in association with the same lineage of Hemlock Woolly Adelgid that is found in the eastern US (Avise 2000, Havill et al. 2006). Since *L. osakensis* may be well-adapted to this particular lineage, its use as a biological control agent is promising (Lamb et al. 2011).

It has been recommended that biological control practitioners increase the probability of adaptation and success of a biological control agent by releasing as much genetic variation as possible into the new region (Phillips et al. 2008, Szűcs et al. 2012). Traits such as climatic adaptation, mate finding, fecundity, mortality, feeding capacity, synchrony with host, habitat preference, and sex ratio are likely to

[1]Department of Entomology, Virginia Tech, 216A Price Hall, Blacksburg, VA 24061. [2]USDA Forest Service Northern Research Station, 51 Mill Pond Road, Hamden, CT 06514. [3]Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario, University of Guelph, 50 Stone Road East, Guelph, ON, Canada. [4]Department of Biological Sciences, 1405 Perry Street, Virginia Tech, Blacksburg, VA 24061. [*]Corresponding author - mfischer@tfs.tamu.edu.

Manuscript Editor: Robert Jetton

affect establishment or control by biological control agents (Hopper et al. 1993). The amount of genetic variation in the pool of *L. osakensis* released for biological control is of particular interest because Hemlock Woolly Adelgid is established across a broad latitudinal range, from Maine to Georgia. Therefore, a biological control agent with the potential of adapting to the climates within this range would be advantageous. Additionally, maintaining high genetic variation by periodically adding new field-collected material from the native range could help avoid inbreeding depression resulting from low effective-population size in the lab (Phillips et al. 2008).

Laricobius osakensis was granted release from quarantine in 2010 by the USDA, Animal and Plant Health Inspection Service (APHIS). However, after sequencing DNA barcodes for members of the *L. osakensis* colony in the fall of 2011, it was discovered that the colony was contaminated by another Japanese species, *Laricobius naganoensis* Leschen. APHIS states in its Plant Health, Plant Protection and Quarantine (PPQ) permit 526 that insects shipped from abroad must not contain unauthorized species; therefore the presence of *L. naganoensis* within the *L. osakensis* colony resulted in the placement of the *L. osakensis* colony back into quarantine before beetles were released in the field.

Laricobius naganoensis is a recently discovered species that was collected from high-altitude areas of Nagano Prefecture where it is sympatric with *L. osakensis* (Leschen 2011). The morphological similarity between the two species and variation within *L. osakensis* make it difficult to differentiate these species. *Laricobius naganoensis* is distinguished from *L. osakensis* by having pale tibiae, more vivid orange-red coloration of the elytra, and an acute median lobe of the male genitalia (Leschen 2011). Females cannot be reliably differentiated using morphology. Males can be differentiated by their genitalia but this identification requires dissection of dead specimens.

After the discovery of *L. naganoensis* contamination in the colony, we established a purification protocol whereby we divided the colony into small groups whose progeny were kept separate. Once oviposition was completed for the season, we planned to determine the identity of the parents to see if there was contamination in each group. We planned to discard the progeny from contaminated groups and retain the progeny of pure groups. Because *L. osakensis* was to be released in the fall of 2012, we needed a quick and inexpensive assay to identify the parental species within the colony. In addition, we evaluated non-lethal DNA extraction methods that might allow genetic analysis to be performed before adults died.

Materials and Methods

To survey genetic diversity, beetle specimens were collected from 2006 through 2011 from nine prefectures in Japan (Tochigi, Nagano, Gunma, Yamanashi, Miyazaki, Nara, Hyogo, Osaka, and Kochi; Appendix 1). Forty-seven *L. naganoensis* specimens were collected from Japan and one *L. naganoensis* was a lab-reared F_1. *Laricobius osakensis* specimens included 121 individuals collected in Japan, as well as 260 lab-reared F_1 individuals. Specimens were preserved in 95–100% ethanol. We used the following methods to process all specimens with the exception

of 93 of the 260 F_1 *L. osakensis*, which were treated using standard DNA barcoding methods at the Canadian Centre for DNA Barcoding (Ivanova et al. 2006). We extracted DNA from beetle thoraces, legs, and second wings using the DNA IQ extraction kit (Promega, Madison, WI), or the DNAeasy kit (Qiagen Inc., Valencia, CA). We eluted samples with 20–30 µl of buffer. We retained heads, elytra, and genitalia as vouchers and deposited them in Yale University's Peabody Museum of Natural History, New Haven, CT.

We amplified the 5' end of the mitochondrial cytochrome *c* oxidase subunit I (COI) using forward primer LepF1 and reverse primer LepR1 (Hebert et al. 2004). PCR was performed in 30-µL reactions containing 3.0 µL 10X PCR Buffer, 2.4 µL dNTPs (10 mM), 4.8 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 (New England Biolabs, Ipswich, MA), and 1.0 µL 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. We purified PCR products using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) or digested them with exonuclease I and antarctic phosphatase (New England Biolabs, Ipswich, MA). Sequencing reactions were performed using the BigDye Terminator kit (Applied Biosystems, Foster City, CA) and analyzed on an Applied BioSystems 3730xl automated sequencer. We aligned sequences using the SeqMan Pro program in Lasergene 8.0 (DNASTAR; http://www.dnastar.com) and calculated sequence divergence (uncorrected p-distance) within and between species using PAUP* (Swofford 2003). We determined diagnostic sites that distinguish the two species by examining sequences in Mesquite 2.75 (Maddison and Maddison 2011). Separate haplotype networks for Japanese wild-caught *L. osakensis* and *L. naganoensis* were reconstructed following the statistical parsimony method of Templeton et al. (1992) using the software TCS 1.21 (Clement et al. 2000, Crandall 1994, Crandall et al. 1994). Haplotype diversity, nucleotide diversity, Tajima's D, and Fu's F_S were calculated for wild *L. osakensis* and wild *L. naganoensis* using Arlequin 3.5 (Excoffier et al. 2005). For each species, accumulation curves showing how the number of haplotypes increased with sample size were estimated with EstimateS 9.1.0 (Colwell 2013) using one hundred randomized runs.

We examined restriction sites within a preliminary set of *L. osakensis* and *L. naganoensis* sequences using Biology WorkBench 3.2 (Subramaniam 1998) or Geneious 5.6.5 (Drummond et al. 2011), resulting in the selection of three possibly diagnostic restriction enzymes: AluI, MboII, and BclI. We used 10 individuals of each species to validate polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) assays using these three enzymes. COI was amplified using the PCR conditions described above. We added 12.5 µL of PCR product to each of the three RFLP reactions containing 0.5 µL of AluI and 1.5 µL of 10X Buffer 4, 0.5 µL of MboII, and 1.5 µL of 10X Buffer 4, or 0.5 µL of BclI and 1.5 µL of 10X Buffer 3 (New England Biolabs, Ipswich, MA). We incubated reactions for 4 h at 37 °C for AluI and MboII, and at 50 °C for BclI, followed by gel electrophoresis and visualization on a 2% agarose gel. We used the software, Geneious, to generate

virtual gels showing all of the unique RFLP patterns from among all the samples that were sequenced.

We chose a subset of the samples used to sequence the COI gene (22 F_1 lab-reared and 12 wild-caught *L. osakensis*, and one lab-reared F_1 and nine wild-caught *L. naganoensis*) to amplify and sequence the internal transcribed spacer ITS2 gene. In contrast to COI, which is strictly maternally inherited, ITS2 is a multi-copy nuclear region, so F_1 hybrids contain copies from both parents. ITS2 was amplified using the primers ITS3 and ITS4 (White et al. 1990) with the same conditions as for COI, but with an annealing temperature of 50 ºC. Sequences were aligned using Muscle 3.6 (Edgar 2004), and a neighbor-joining tree was constructed using PAUP*, with uncorrected p-distance, indels not scored as characters, and *Laricobius kangdingensis* Zilahi-Balogh and Jelinek (Genbank Assession HQ433487) as an outgroup. We deposited all new sequences generated from this study in GenBank (Appendix 1).

We used *Laricobius osakensis* specimens to explore whether we could extract DNA using non-lethal methods. Beetles had been preserved in 95% ethanol at -20 °C and then air-dried before use. We removed a single antenna and a single tarsus from five specimens. Because using antennae appeared to yield better results (see results), we completed an additional experiment using 20 *L. osakensis* antennae to further test the success rate. Each PCR reaction contained 6.25 µL of 10% D- (+)-trehalose dihydrate (Fluka Analytical), 1.25 µL of 10X PCR buffer, 0.625 µL $MgCl_2$ (50 mM), 0.0625 µL of each 10 µM primer (LepF1, LepR1, LCO1490, HCO2198; Folmer et al. 1994), 0.0625 µL of dNTPs (10mM), 0.060 µL of 5 U/µL PlatinumTaq DNA Polymerase (Invitrogen), and 2.0 µL of DNA template for a total reaction volume of 12.5 µL. Thermal-cycling conditions were 94 °C for 1 min, followed by 5 cycles of 94 °C for 40 s, 45 °C for 40 s, 72 °C for 1 min, then 35 cycles of 94 °C for 40 s, 51 °C for 40 s, 72 °C for 1 min, and a final extension of 72 °C for 5 min.

In 2012, we split the *L. osakensis* colony into eight groups of approximately 20 adult beetles whose progeny were kept separate. Our goal was to purify the colony by separating *L. naganoensis* male and female beetles and prevent them from reproducing. After beetles had completed oviposition and died, we identified all parents to species. We dissected the beetles and identified males by using genital morphology and females by using the AluI and MboII RFLP assays described above.

Results

We cropped all *Laricobius* COI sequences to 658 bp-long; their alignment required no gaps, and amino acid translation contained no stop codons. P-distance (proportion of nucleotide differences between sequences) within *L. osakensis* ranged from 0 to 2.12% with a mean of 0.74% for wild-collected specimens and a range of 0–1.97% with a mean of 0.79% for F_1 colony-collected specimens. P-distance within *L. naganoensis* ranged from 0 to 2.28% with a mean of 1.11%. P-distance between *L. osakensis* and *L. naganoensis* ranged from 7.33 to 9.45% with a mean of 8.35%. There are 21 diagnostic CO1 nucleotide sites (fixed differences

between species) spread throughout the 658-bp COI barcode region. The haplotype network for wild-caught *L. osakensis* exhibited very high diversity with 90 unique haplotypes in the 121 individuals sampled (Fig. 1). Of the 90 sites with nucleotide substitutions, 13 were at the first codon position, one at the second codon position, and 76 at the third codon position. All were synonymous (did not result in amino acid changes) with the exception of substitutions in one individual each at positions 83, 200, 263, 506, 515, and 640, and in three individuals at position 491. Haplotype diversity (H) was 0.989, and nucleotide diversity was 0.008. Tajima's D, and Fu's F_S were -2.209 and -25.324, respectively, and both were significantly different from neutral expectations ($p < 0.01$). The COI network for wild *L. naganoensis* exhibited high haplotype diversity as well, with 32 unique haplotypes of the 47 wild individuals sampled (Fig. 1). Of the 39 sites with nucleotide substitutions, four were at the first codon position, one at the second codon position, and 34 at the third codon position. All were synonymous substitutions with the exception of a substitution in one individual at position 318. For *L. naganoensis,* haplotype diversity (H) was 0.9628, and nucleotide diversity was 0.011. Tajima's D, and Fu's F_S were -0.7109 and -17.044, respectively, and both were significantly different from neutral expectations ($P < 0.01$). Neither of the accumulation curves for either species showed evidence of reaching an asymptote, and therefore, they indicate that all of the diversity has not been sampled for these species (Fig. 2).

Two of the enzymes (AluI and MboII) can be used to correctly identify all samples of *L. osakensis* and *L. naganoensis* that have been collected to date. Digestion by AluI produced four fragment patterns for *L. naganoensis*, and ten patterns

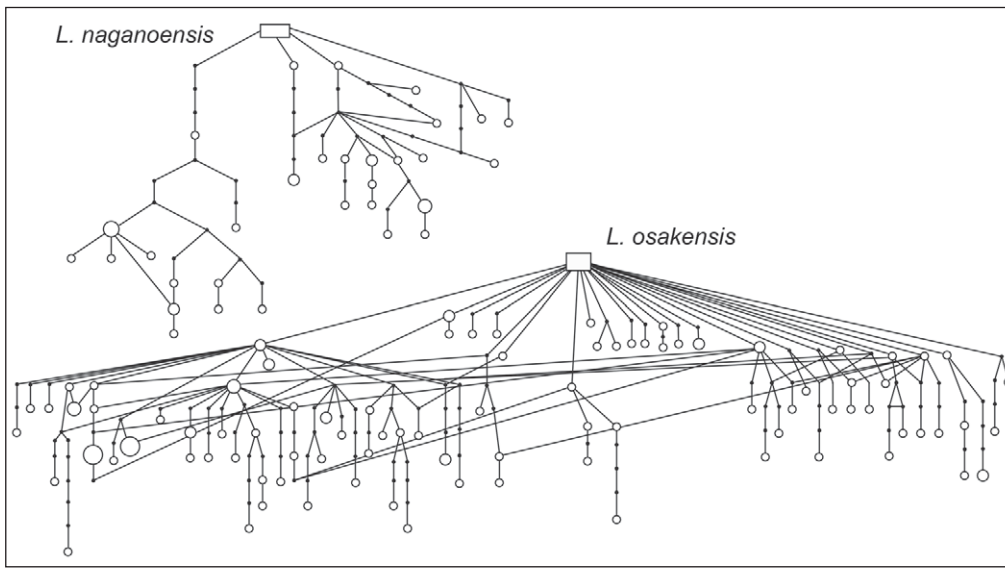

Figure 1. TCS networks showing relationships among COI haplotypes for wild-caught *Laricobius naganoensis* (47 individuals, 32 unique haplotypes) and wild-caught *L. osakensis* samples (121 individuals, 90 unique haplotypes). The squares denote the inferred ancestral haplotypes. The size of each shape is proportional to the frequency of the haplotype. Small black dots represent unsampled intermediate haplotypes.

for *L. osakensis* (Fig. 3A). All of the patterns for *L. osakensis* contained a fragment larger than 201 bp, distinguishing them from *L. naganoensis*. Digestion by MboII produced one pattern for *L. naganoensis* and three patterns for *L. osakensis* (Fig. 3B). Similar to AluI, *L. osakensis* can be distinguished from *L. naganoensis* by the presence of at least one fragment greater than 170 bp. Digestion by BclI produced two patterns for *L. naganoensis* and three patterns for *L. osakensis* (Fig. 3C). There was one *L. naganoensis* individual that shared a pattern with *L. osakensis*, so identification using this enzyme is not always correct.

Length of ITS2 sequences were 402 bp for all *L. osakensis* specimens and 397 bp for all *L. naganoensis* specimens. The alignment resulted in three indels that were 1 bp long and one indel that was 2 bp long. All chromatograms were free of superimposed peaks, which would have indicated amplification of different-sized ITS2 copies. The neighbor-joining tree (Fig. 4) showed a clear separation between the species with no evidence of hybridization.

For the two potentially non-lethal treatments tested, sequences were successfully recovered from all five antennae and from two of five tarsi. The second experiment using 20 *L. osakensis* produced full-length 658-bp sequences that matched *L. osakensis* for all samples.

After splitting the colony into eight small groups and testing the parental beetles, we determined that six groups contained *L. naganoensis* males and females, precluding their use for the colony because of possible contamination in the next generation (Table 1). One group (SK6) contained *L. naganoensis* females but no males. We also removed this group from the colony because the females could have mated in the field prior to being collected. One group (SK1) contained only one male *L. naganoensis;* we removed this group from quarantine and used it to start the next generation.

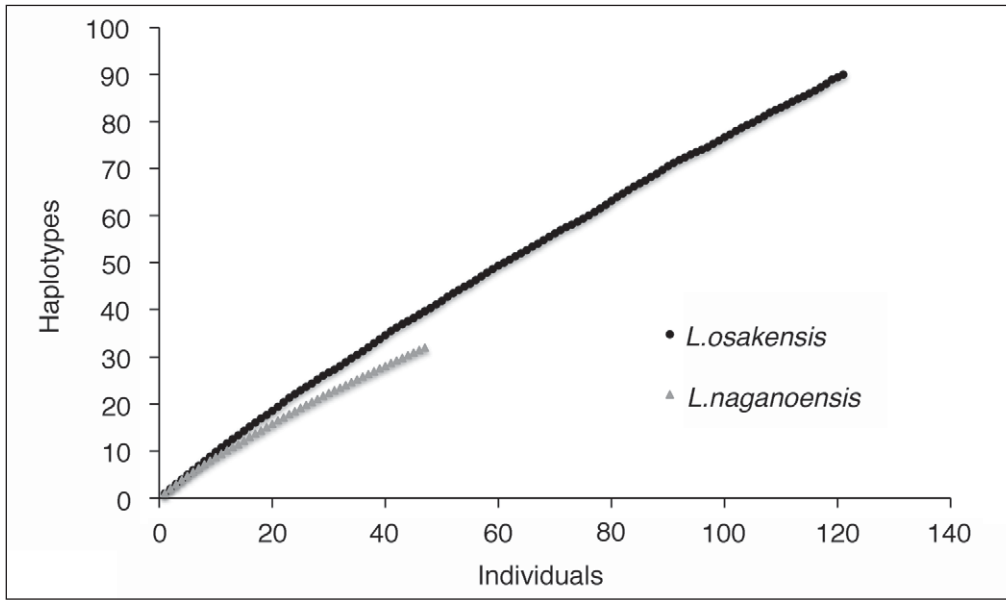

Figure 2. Accumulation curves showing how the number of haplotypes increase with the number of individuals sampled for *Laricobius osakensis* and *L. naganoensis*.

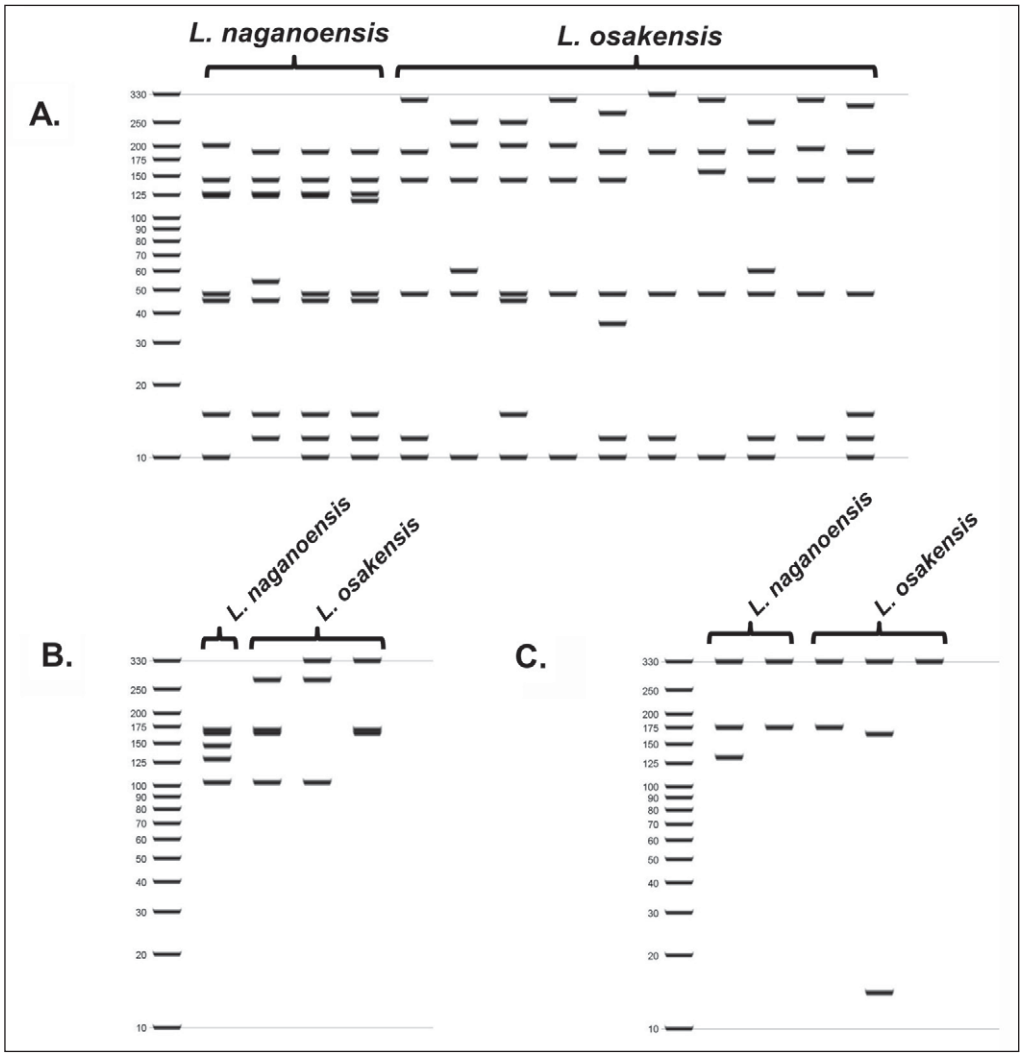

Figure 3. Virtual agarose gel electrophoreses of *Laricobius naganoensis* and *L. osakensis* COI PCR products digested with: A) AluI, B) MboII, and C) BclI. The first lane is a simulated 10-bp ladder.

Table 1. Species and sex of parent beetles in eight colony-rearing groups.

	Laricobius osakensis		*L. naganoensis*	
Group	Female	Male	Female	Male
SK1	12	10	0	1
SK2	11	7	4	2
SK3	8	7	4	4
SK4	14	8	4	3
SK5	12	8	3	1
SK6	12	10	3	0
SK7	9	11	1	1
NS1	5	8	3	2

Discussion

Laricobius naganoensis and *L. osakensis* are not closely related (p-distance = 8.35%), and our results from sequencing ITS2 showed no evidence of hybridization in the field or in the lab colony. It is therefore unlikely that the two species can hybridize, an event that has occurred in recent biological control efforts involving other *Laricobius* spp. (Fischer 2013, Havill et al. 2012). Two closely related North American species, *L. nigrinus* and *L. rubidus*, which exhibit approximately 2% COI sequence divergence were found to readily hybridize, while *L. nigrinus* and *L. osakensis* with 12% divergence separating them did not (Fischer 2013).

Recent population expansion is implied by high haplotype diversity, low nucleotide diversity, negative values for both Tajima's D test and Fu's F_s test, as well as a haplotype network showing low levels of sequence divergence and a high frequency of unique mutations (Avise 2000, Halliburton 2004). These patterns were

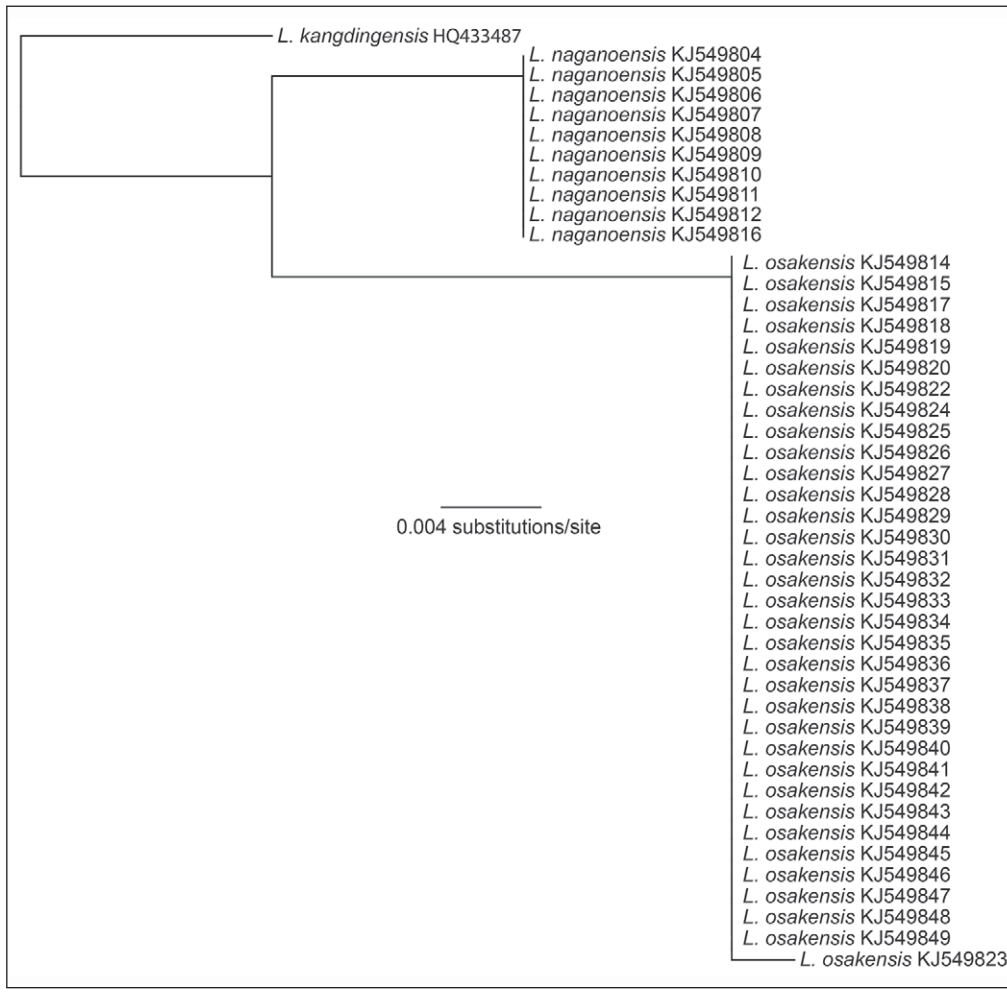

Figure 4. Neighbor-joining tree of nuclear ITS2 sequences showing separation of *Laricobius oakensis* and *L. naganoensis* with no evidence of hybridization.

evident for both Japanese *Laricobius* species. Recent population expansion following the last glacial period is a pattern that has been observed in several organisms in Japan and throughout the northern hemisphere (Hewitt 2000, Kawamoto et al. 2007, Liu et al. 2006). Climatic conditions during the last glaciation reduced the available habitat for many species through contraction of forests (Hewitt 2000, Tsukada 1983). Following this period, evidence suggests that the distributional range of many organisms expanded along with available habitat (Hewitt 2000, Kawamoto et al. 2007, Liu et al. 2006).

Almost all of the intra-specific nucleotide substitutions in our data set were neutral (synonymous) and therefore the diversity that we document is a reflection of natural diversity, not due to PCR error or base-calling mistakes. The extensive mitochondrial variation within *L. osakensis* and *L. naganoensis* as well as their haplotype accumulation curves suggest that we have documented only a small amount of the variation within these species. Continued collections of both species in Japan and genotyping of new rearing stock will be necessary to fully characterize the diversity within both species. This high diversity should allow *L. osakensis* to adapt to new environments, which is advantageous for a biological control agent. Colony maintenance and augmentation should seek to maintain this high diversity.

Without the proper permits, *L. naganoensis* cannot be released legally in the US. Therefore, distinguishing between *L. osakensis* and *L. naganoensis* is currently necessary for universities and state or federal agencies that will be importing *L. osakensis* from Japan for biological control of Hemlock Woolly Adelgid. The RFLP assay developed here is less expensive and less time consuming than DNA sequencing, and the equipment needed for this assay is available in most basic molecular labs. The enzymes AluI and MboII were each sufficient for distinguishing the species. However, since there is likely to be more natural diversity than we have sampled to date, possibly resulting in additional banding patterns, we recommend using both enzymes independently and sequencing any individuals for which the assay results do not match or which produce new gel patterns not reported here.

Although only one of the eight *L. osakensis* groups was found to be pure following RFLP, this one group produced approximately 200 adult progeny, which are currently being mass reared for releases in fall 2014. This result demonstrates that purification can work in an applied setting, and that even with a small starting colony (approximately 20 beetles), mass rearing can be successful.

Our results show that a single antenna from a *Laricobius* specimen is sufficient for recovery of a full-length 658-bp COI barcode sequence. Several samples consisted of less than an entire antenna; one consisted of only two segments, suggesting that *Laricobius* antennae are mtDNA-rich and an excellent tissue source for non-lethal barcoding. A study on non-lethal sampling using antennae of Japanese diving beetles had similar results (Suzuki et al. 2012); furthermore, they found that removal of an antenna did not affect mating, oviposition, or lifespan. Additional experiments using live *Laricobius* specimens are necessary to determine if the removal of a single antenna will impact beetle survival and reproduction in the lab

and whether a non-lethal approach is more cost-effective than identifying beetles after they have died.

Acknowledgments

This work was supported by USDA Forest Service Agreements 11-CA-11420004-132 and 11-CA-11420004-133. Work at the Biodiversity Institute of Ontario was aided by a grant from Genome Canada through the Ontario Genomics Institute. Technical assistance was provided by DeAdra Newman, Brenna Traver, Stephanie Vece, and Jacquelyn Martin. We would like to thank those individuals who assisted in the collection of specimens.

Literature Cited

Avise, J.C. 2000. Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge, MA. 447 pp.

Clement, M., D. Posada, and K. Crandall. 2000. TCS: A computer program to estimate gene genealogies. Molecular Ecology 9:1657–1659.

Crandall, K.A. 1994. Intraspecific cladogram estimation: Accuracy at higher levels of divergence. Systematic Biology 43:222–235.

Crandall, K., A. Templeton, and C. Sing. 1994. Intraspecific phylogenetics: Problems and solutions. Pp. 273–298, *In* R. Scotland, D.J. Siebert, and D.M. Williams (Eds.). Models in Phylogeny Reconstruction. Oxford University Press, New York, NY.

Drummond, A., B. Ashton, S. Buxton, M. Cheung, A. Cooper, C. Duran, M. Field, J. Heled, M. Kearse, S. Markowitz, R. Moir, S. Stones-Havas, S. Sturrock, T. Thierer, and A. Wilson. 2011. Geneious v5.4. Available online at http://www.geneious.com/. Accessed 22 April 2013.

Edgar, R.C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32**:**1792–1797.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1:47–50.

Fischer, M.J. 2013. Evaluation of hybridization among three *Laricobius* species, predators of Hemlock Woolly Adelgid (Adelgidae). Ph.D Dissertation. Virginia Polytechnic Institute and State University, Blacksburg, VA. 163 pp.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3:294–299.

Halliburton, R. 2004. Introduction to Population Genetics. Pearson Prentice Hall, Upper Saddle River, NJ. 650 pp.

Havill, N.P., M.E. Montgomery, G. Yu, S. Shiyake, and A. Caccone. 2006. Mitochondrial DNA from Hemlock Woolly Adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. Annals of the Entomological Society of America 99:195–203.

Havill, N.P., G.A. Davis, J. Klein, D. Mausel, R. McDonald, C. Jones, M.J. Fischer, S. Salom, and A. Caccone. 2012. Hybridization between a native and introduced predator of Adelgidae: An unintended result of classical biological control. Biological Control 63:359–369.

Hebert, P.D.N., E.H. Penton, J.M. Burns, D.H. Janzen, and W. Hallwachs. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proceedings of the National Academy of Sciences 101:14,812–14,817.

Hewitt, G. 2000. The genetic legacy of the Quaternary ice ages. Nature 405:907–913.

Hopper, K., R. Roush, and W. Powell. 1993. Management of genetics of biological-control introductions. Annual Review of Entomology 38:27–51.

Ivanova, N.V., J.R. Dewaard, and P.D.N. Hebert. 2006. An inexpensive, automation-friendly protocol for recovering high-quality DNA. Molecular Ecology Notes 6:998–1002.

Kawamoto, Y., T. Shotake, K. Nozawa, S. Kawamoto, K.-i. Tomari, S. Kawai, K. Shirai, Y. Morimitsu, N. Takagi, H. Akaza, H. Fujii, K. Hagihara, K. Aizawa, S. Akachi, T. Oi, and S. Hayaishi. 2007. Postglacial population expansion of Japanese Macaques (*Macaca fuscata*) inferred from mitochondrial DNA phylogeography. Primates 48:27–40.

Lamb, A.B., M.E. Montgomery, L.C. Vieira, S. Shiyake, and S. Salom. 2011. *Laricobius osakensis*, a Hemlock Woolly Adelgid predator from Japan. Pp. 90–96, *In* B. Onken and R.C. Reardon (Eds.). Implementation and Status of Biological Control of the Hemlock Woolly Adelgid. US Department of Agriculture Forest Service, Morgantown, WV.

Leschen, R.A.B. 2011. World review of *Laricobius* (Coleoptera: Derodontidae). Zootaxa 2908**:**1–44.

Liu, J.-X., T.-X. Gao, Z.-M. Zhuang, X.-S. Jin, K. Yokogawa, and Y.-P. Zhang. 2006. Late Pleistocene divergence and subsequent population expansion of two closely related fish species, Japanese Anchovy (*Engraulis japonicus*) and Australian Anchovy (*Engraulis australis*). Molecular Phylogenetics and Evolution 40:712–723.

Maddison, W., and D. Maddison. 2011. Mesquite: A modular system for evolutionary analysis. Version 2.75. Available online at http://mesquiteproject.org. Accessed 17 April 2013.

Phillips, C., D. Baird, I. Iline, M. McNeill, J. Proffitt, S. Goldson, and J. Kean. 2008. East meets west: Adaptive evolution of an insect introduced for biological control. Journal of Applied Ecology 45:948–956.

Subramaniam, S. 1998. The Biology Workbench: A seamless database and analysis environment for the biologist. Proteins 32:1–2.

Suzuki, G., T. Inoda, and S. Kubota. 2012. Nonlethal sampling of DNA from critically endangered diving beetles (Coleoptera: Dytiscidae) using a single antenna. Entomological Science 15:352–356.

Swofford, D. 2003. PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4. Sinauer Associates, Sunderland, MA.

Szűcs, M., U. Schaffner, W.J. Price, and M. Schwarzländer. 2012. Post-introduction evolution in the biological control agent *Longitarsus jacobaeae* (Coleoptera: Chrysomelidae). Evolutionary Applications 5:858–868.

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

Tsukada, M. 1983. Vegetation and climate during the last glacial maximum in Japan. Quaternary Research 19:212–235.

White, T.J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315–322, *In* M. Innis, D. Gelfand, J. Sninsky, and T. White (Eds.). PCR Protocols: A Guide to Methods and Applications. Academic, San Diego, CA. 315–322 pp.

Appendix 1. Specimen data. *n* = number of specimens.

Genbank Accession No.				
COI	ITS	*n*	Species	Collection information
JX871945	KJ549809	1	*Laricobius naganoensis*	Japan; Nagano Prefecture; Fujimi; Mt. Nyukasa; 1 June 2008; Coll. N.P. Havill, S. Shiyake, A. Lamb
JX872066 and JX872067	KJ549806, KJ549808	2	*L. naganoensis*	Japan; Nagano Prefecture; Matsumoto; Mt. Norikura; 15 October 2008; S. Shiyake, A. Lamb
JX872068-JX872070	KJ549807, KJ549811	3	*L. naganoensis*	Japan; Nagano Prefecture; Ootaki; Mt. Ontake; 29 October 2009; A. Lamb, S. Shiyake
JX872071	KJ549812	1	*L. naganoensis*	Japan; Nagano Prefecture; Yamanouchi; Shiga-kogen; 31 October 2010; A. Lam, S. Shiyake, C. Jubb
JX872072-JX872075	KJ549804, KJ549805, KJ549810	4	*L. naganoensis*	Japan; Gunma Prefecture; Katashina; Mt. Nikko-Shirane; Marunuma-Kogen Ski Hills; 31 October 2010; A. Lamb, S. Shiyake, C. Jubb
JX871946		1	*L. naganoensis*	Virginia Tech Department of Entomology Insect Rearing Facility; October 2010; M. Fischer
KC182379	KJ549816	1	*L. naganoensis*	Virginia Tech Department of Entomology Insect Rearing Facility; December 2011; C. Jubb
KJ187715-KJ187744		30	*L. naganoensis*	Japan; Nagano Prefecture; Yamanouchi; Shiga-kogen; 4 November 2012; Lamb, S. Shiyake, C. Jubb
KJ187710-KJ187714		5	*L. naganoensis*	Japan; Gunma and Nagano Prefectures; November 2012; A. Lamb, S. Shiyake, C. Jubb
HM803301-HM803307	KJ549814, KJ549820	7	*L. osakensis*	Virginia Tech Department of Entomology Insect Rearing Facility; November 2006; A. Lamb
HM803466		1	*L. osakensis*	Japan; Hyogo Prefecture; Kobe; Arima Onsen; 7 January 2008; S. Shiyake, A. Lamb
HM803464 and HM803465		2	*L. osakensis*	Japan; Kochi Prefecture, Tosa-Yamada; Hokigamine Forest Park; 6 January 2008; S. Shiyake, A. Lamb
HM803461		1	*L. osakensis*	Japan; Hyogo Prefecture; Kobe; Kobe Municipal Arboretum; 9 January 2008; S. Shiyake, A. Lamb
HM803463		1	*L. osakensis*	Japan; Osaka Prefecture; Takatsuki; Nakahata; 9 January 2008; S. Shiyake, A. Lamb
HM803467		1	*L. osakensis*	Japan; Hyogo Prefecture; Kobe; Kobe Municipal Arboretum; 14 January 2008, A. Lamb

Genbank Accession No.				
COI	ITS	*n*	Species	Collection information
HM803462		1	*L. osakensis*	Japan; Nara Prefecture; Nara Park; Wakakusa-Yama; 18 January 2008; A. Lamb
JX872076 and JX872077		2	*L. osakensis*	Japan; Miyazaki Prefecture; Gokase; Gokase Ski Hills; 26 May 2008, N.P. Havill, S. Shiyake, A. Lamb
JX872078 and JX872079		2	*L. osakensis*	Japan; Nagano Prefecture, Fujimi, Mt. Nyukasa, 1 June 2008, N.P. Havill, S. Shiyake, A. Lamb
JX872080 and JX872081		2	*L. osakensis*	Japan; Yamanashi Prefecture, Hokuto; Yokote; Yokote Shrine, 2 June 2008; N.P. Havill, S. Shiyake, A. Lamb
HM803616		1	*L. osakensis*	Japan; Nagano Prefecture, Shigakogen, Yamanouchi, 6 May 2009, S. Shiyake, A. Lamb
HM803617		1	*L. osakensis*	Japan, Nagano Prefecture, Mt. Norikura, Matsumoto, 4 April 2009, S. Shiyake, A. Lamb
HM803618		1	*L. osakensis*	Japan; Tochigi Prefecture; Nikko; Konsei Pass; 3 April 2009; S. Shiyake, A. Lamb
HM803681 and HM803682		2	*L. osakensis*	Japan; Nagano Prefecture; Mt. Norikura; Matsumoto; 28 October 2009; A. Lamb, S. Shiyake
HM803683		1	*L. osakensis*	Japan; Tochigi Prefecture; Nikko; Konsei Pass; 1 November 2009; A. Lamb, S. Shiyake
HM803684		1	*L. osakensis*	Japan, Nagano Prefecture, Yamanouchi; Shiga-kogan; 30 October 2009; A. Lamb, S. Shiyake
HM803685		1	*L. osakensis*	Japan, Tochigi Prefecture; Nikko; Nikko-Yumoto Spa; 31 October 2009; A. Lamb, S. Shiyake
JX872082-JX872084		3	*L. osakensis*	Japan; Gunma Prefecture; Katashina; Mt. Nikko-Shirane; Marunuma-Kogen Ski Hill; 31 October 2009; S. Shiyake, A. Lamb
JX872085 and JX872086		2	*L. osakensis*	Japan; Nagano Prefecture; Yamanouchi; Shiga-kogen; 30 October 2009; S. Shiyake, A. Lamb
JX871947-JX871960		14	*L. osakensis*	Virginia Tech Department of Entomology Insect Rearing Facility; December 2009; M. Fischer, N. Morris
JX871961-JX872041		81	*L. osakensis*	Virginia Tech Department of Entomology Insect Rearing Facility; October 2010; M. Fischer, P. Thomasson
JX872042-JX872065		24	*L. osakensis*	Japan; October 2010; A. Lamb, S. Shiyake, C. Jubb

Genbank Accession No.				
COI	ITS	*n*	Species	Collection information
JX872087–JX872111	KJ549817, KJ549824, KJ549830, KJ549833, KJ549842, KJ549846	25	*L. osakensis*	Japan; Nagano Prefecture; Yamanouchi; Shiga-kogen; 31 October 2010; A. Lamb, S. Shiyake, C. Jubb
JX872112–JX872130	KJ549819, KJ549825, KJ549827, KJ549838, KJ549839, KJ549849	19	*L. osakensis*	Japan; Gunma Prefecture; Katashina; Mt. Nikko-Shirane; Marunuma-Kogen Ski Hills; 31 October 2010; A. Lamb, S. Shiyake, C. Jubb
JX872131–JX872157		27	*L. osakensis*	Japan; Tochigi Prefecture; Nikko; Yumoto Spa; 31 October 2010; A. Lamb, S. Shiyake, C. Jubb
JX872215–JX872222		8	*L. osakensis*	Virginia Tech Department of Entomology Insect Rearing Facility; October 2011; M. Fischer, M. Cassell
JX872210–JX872214	KJ549822, KJ549834, KJ549835, KJ549840, KJ549841	4	*L. osakensis*	Virginia Tech Department of Entomology Insect Rearing Facility; 28 October 2011; M. Fischer
JX872158–JX872209, KC182381–KC182473, KC412635–KC412661	KJ549815, KJ549818, KJ549823, KJ549826, KJ549828, KJ549829, KJ549831, KJ549832, KJ549836, KJ549837, KJ549843, KJ549844, KJ549845, KJ549847, KJ549848	173	*L. osakensis*	Virginia Tech Department of Entomology Insect Rearing Facility; December 2011; C. Jubb