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Evolution

Prey-associated genetic differentiation in two species of silver fly (Diptera: Chamaemyiidae), *Leucotaraxis argenticollis* and *L. piniperda*

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Sympatric host-associated genetic differentiation is a prominent pattern that could lead to speciation. In insects, there are numerous examples of host-associated differentiation among herbivores that prefer different plants, and parasitoids that prefer different hosts, but few examples for specialist predators. We developed new microsatellite loci for two species of silver fly, *Leucotaraxis argenticollis* (Zetterstedt) and *L. piniperda* (Malloch) (Diptera: Chamaemyiidae), being evaluated as biological control agents for the hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), in eastern North America where it is a nonnative pest. We obtained DNA from specimens of both fly species feeding on native *A. tsugae* in western North America, as well as on other western and eastern adelgid species. We performed population genetic analyses using the new loci and DNA barcode sequences. Our results confirmed east–west allopatric divergence and uncovered nested genetic differentiation associated with different adelgid prey species and their host plants in western North America for both species of silver flies. For both species, there is also evidence for a longer history of diversification in the west, with ancestral specialization of feeding on pine adelgids, which was retained after range expansion to the east. More recently, divergence to feeding on new adelgid prey species occurred in the west. Our findings are consistent with the hypothesis that host-alternating life cycles in Adelgidae may provide temporary escape from specialist predators. We discuss the implications for biological control efficacy and potential for lineage hybridization as western flies are released in the east to control *A. tsugae*.

Key words: microsatellite, genetic structure, specialist predator, invasive species, hemlock woolly adelgid

Introduction

Understanding the factors that generate biodiversity is one of the foundations of evolutionary biology, but much remains unknown about the patterns and processes that drive speciation (Mallet 2005). Most of the earliest examples demonstrated that speciation was the result of allopatric processes, where populations diverged in geographically isolated locations ultimately resulting in the formation of distinct species (Mayr 1942, Futuyma and Mayer 1980). More recently, sympatric speciation has been recognized to be an important process whereby selection associated with distinct ecological factors promotes population divergence without geographic isolation (Nosil 2012). Indeed, correlations between genetic differentiation and environmental factors are widespread across taxa (Shafer and Wolf 2013). Of these, host-associated differentiation is a prominent pattern that could lead to speciation, wherein the specialization of separate lineages on distinct coexisting hosts drives

the speciation process (Berlocher and Feder 2002, Drès and Mallet 2002, Matsubayashi et al. 2010). In these cases, reproductive isolation can develop when divergent selection leads to reduced gene flow and eventually to the evolution of reproductive barriers (Erlich and Raven 1969, Bush 1994, Via 2001, Nosil 2012).

For insects in particular, there have been numerous examples of sympatric host-associated genetic differentiation among herbivores that feed on different plant species (e.g., Via 1999; Forbes et al. 2009, Peccoud and Simon 2010, Hood et al. 2015, Bakovic et al. 2019, Driscoe et al. 2019), and among parasitoids that prefer different hosts (e.g., Hoffmeister 1992, Abrahamson and Blair 2008, Forbes et al. 2009, Hood et al. 2015). However, there are few examples of sympatric host-associated differentiation for insect predators (e.g., Tauber et al. 1993, Eubanks et al. 2003, Noriyuki and Osawa 2016). Like parasitoids, predatory insects often locate their herbivorous prey using chemical and/or visual cues either from the prey or from the prey's host plants (Vet and Dicke 1992). It is therefore surprising that it is common to observe genetic differentiation associated with parasitoids' ability to locate hosts on different plants, while the same pattern is rarely observed for predators (Thompson et al. 2022).

Although there is a tendency for predators to have a wider phylogenetic host range than parasitoids, many are known to specialize on one or only a few closely related prey species (Strand and Obrycki 1996). For pests that do not have specialized parasitoids, specialized predators have been evaluated as potential biological control agents of nonnative herbivorous pests (Van Driesche and Winston 2022). The recognition that populations of candidate biological control agents may be undergoing host-associated differentiation has the potential to increase the efficacy and safety of biological control programs by shifting the focus to intraspecific genetic lineages (sometimes referred to in the biological control literature as strains, ecotypes, races, or biotypes) to ensure that predators will specialize on their intended target pests (Thompson et al. 2022).

Predators have been the focus of biological control programs for adelgid (Hemiptera: Adelgidae) pests (Havill et al. 2016) because they have no known parasitoids and no widespread diseases (Havill and Foottit 2007). *Adelges tsugae* Annand is a nonnative pest of hemlock trees, *Tsuga* (Pinales: Pinaceae), in eastern North America (Limbu et al. 2018). There are multiple genetically distinct lineages of *A. tsugae* that are native to different regions of Asia and in western North America, and the lineage introduced to eastern North America came from southern Japan (Havill et al. 2016). Recently, western North America has been the primary region of exploration for potential natural enemies because the western lineage of *A. tsugae* is closely related to the southern Japanese lineage (Havill et al. 2016). A beetle species, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), and two silver fly species, *Leucotaraxis argenticollis* (Zetterstedt) and *L. piniperda* (Malloch) (Diptera: Chamaemyiidae), are among the most abundant specialist predators of *A. tsugae* in western North America (Kohler et al. 2008, 2016, Rose et al. 2020). *Laricobius nigrinus* has been released in large numbers in the east, and has become widely established, reaching high enough numbers that it can be collected in some areas for redistribution in the region (Havill et al. 2016). However, *La. nigrinus* has not exhibited adequate regulation in the eastern U.S., likely because it only feeds on one of the two annual *A. tsugae* generations, allowing populations to rebound (Crandall et al. 2020).

In contrast, the two *Leucotaraxis* species occur throughout the *A. tsugae* life cycle on western hemlock trees (Neidermeier et al. 2020, Dietschler et al. 2021). Distinct peaks of adult emergence suggest that *L. argenticollis* probably lays eggs early in the year, mainly consuming eggs and nymphs of the first *A. tsugae* generation, whereas *L. piniperda* lays eggs later to feed on eggs and nymphs of the second generation. A second emergence of *L. argenticollis* adults occurs at some sites, but this is not well understood (Dietschler et al. 2021). This temporal niche partitioning among *La. nigrinus* and the two *Leucotaraxis* species may contribute to the regulation of *A. tsugae* populations in western North America (Crandall et al. 2022).

Havill et al. (2018) and Gaimari and Havill (2021) documented broad allopatric prey-associated genetic divergence in both *Leucotaraxis* species by reconstructing their phylogenies based on DNA sequence data. Each species had distinct western and eastern North American clades. In the west, both species feed on hemlock woolly adelgid, while in the east, they were only observed feeding on pine adelgids (species of *Pineus* Shimer), indicating that western and eastern populations have different adelgid prey preferences. The preference for pine adelgids over hemlock adelgids in the eastern populations has persisted even after *A. tsugae* arrived as a potential new food source where pines and hemlocks occur together in mixed forests (Montgomery and Lyon 1996, Wallace and Hain 2000). This is probably due to differences in host tree and/or prey location behaviors between the lineages, perhaps using volatile cues, although this has not been specifically investigated in either *Leucotaraxis* species. The genetic differentiation between eastern and western flies, and stark differences in their host associations led to the hope that western flies could be used as biological control agents to help regulate invasive *A. tsugae* populations in the east. Western flies of both *Leucotaraxis* species have since been found to feed and reproduce on Japanese *A. tsugae* on caged branches of eastern hemlock (Motley et al. 2017), and have been released since 2015 in the eastern United States (Virginia Tech 2022).

Previous studies that revealed broad patterns of east–west divergence within each species used methods that were not suitable for examining more fine-scale population genetic patterns. Understanding potential regional patterns of sympatric prey-associated differentiation within eastern and western lineages for each species could have important implications for selecting the most effective populations of silver flies to serve as biological control agents of adelgids. Adelgid species typically have host-alternating life cycles that alternate between generations on *Picea* primary hosts, and secondary hosts in other conifer genera (*Tsuga, Pinus, Abies, Larix*, or *Pseudotsuga*). Patterns of prey-association on alternate hosts could also have implications for understanding the evolution of adelgid life cycles, if host alternation provides temporal escape from specialized natural enemies, as some have suggested (Way and Banks 1968, Havill and Foottit 2007). Patterns of prey association could also help predict the extent to which the different silver fly lineages might hybridize if the western lineages become established in eastern North America.

In this study, we explore patterns of population genetic differentiation within *L. argenticollis* and *L. piniperda* using newly developed microsatellite loci. We included more samples collected from additional adelgid prey than were reported in Havill et al. (2018) and Gaimari and Havill (2021), to examine finer-scale genetic structure. We test whether there is genetic structure associated with adelgid prey species to look for evidence of prey preference and host-associated differentiation. Our data also document baseline genetic diversity in the east to allow for monitoring of potential hybridization between lineages after establishment of the western flies released as biological control agents in the east.

Methods

Sample Collection and DNA Extraction

Silver flies were collected from 55 locations between 2002 and 2021 (Tables 1 and 2, Fig. 1, Supplementary Files S1 and S2). Most immature silver flies were collected by examining bark or twigs of

Table 1. *Leucotaraxis argenticollis* sampling locations. Location number refers to locations indicated in Fig. 1A

Population number	Population name	State/province	Latitude	Longitude	Prey and host	*N* microsatellite	*N* COI
1	Big Lake	Alaska	61.520	−149.983	*Pineus coloradensis* on *Pinus contorta*	1	1
2	Mount Washington	British Columbia	49.746	−125.320	*Adelges tsugae* on *Tsuga mertensiana*	2	3
3	Nanaimo Lakes	British Columbia	49.092	−124.111	*Adelges tsugae* on *Tsuga heterophylla*	2	2
4	Trinidad	California	41.239	−124.084	*Adelges tsugae* on *Tsuga heterophylla*	46	46
5	Waldport	Oregon	44.418	−124.049	*Adelges tsugae* on *Tsuga heterophylla*	1	1
6	Olympia	Washington	47.026	−122.901	*Adelges tsugae* on *Tsuga heterophylla*	48	61
7	San Juan Islands	Washington	48.641	−122.787	*Adelges tsugae* on *Tsuga heterophylla*	56	56
8	Whidbey Island	Washington	48.095	−122.609	*Adelges tsugae* on *Tsuga heterophylla*	64	62
9	Tacoma	Washington	47.367	−122.482	*Adelges tsugae* on *Tsuga heterophylla*	18	13
10	Vancouver	Washington	45.703	−122.671	*Adelges tsugae* on *Tsuga heterophylla*	27	37
11	West Valley	Washington	46.498	−121.184	*Pineus coloradensis* on *Pinus albicaulis*	1	1
12	Deschutes National Forest	Oregon	43.565	−121.177	*Pineus coloradensis* on *Pinus contorta*	5	5
13	Colville National Forest	Washington	48.667	−118.451	*Pineus coloradensis* on *Pinus contorta* and *Pinus monticola*	6	9
14	Colville Reservation	Washington	48.311	−118.202	*Pineus coloradensis* on *Pinus ponderosa*	2	2
15	Coeur d'Alene	Idaho	47.700	−116.775	*Adelges tsugae* on *Tsuga heterophylla*	8	7
16	Boise National Forest	Idaho	43.896	−115.713	*Adelges piceae* on *Abies lasiocarpa*	2	2
17	Choteau	Montana	47.898	−112.572	*Pineus coloradensis* on *Pinus flexilis*	3	3
18	Ogden	Utah	41.372	−111.925	*Adelges piceae* on *Abies lasiocarpa*	5	5
19	Salt Lake	Utah	40.784	−111.711	*Adelges piceae* on *Abies lasiocarpa*	13	14
20	Caribou-Targhee National Forest	Wyoming	43.705	−110.985	*Pineus abietinus* on *Abies lasiocarpa*	5	6
21	Cascade Lake	Wyoming	44.752	−110.486	*Pineus coloradensis* on *Pinus contorta*	1	1
22	Zimmerman Lake	Colorado	40.540	−105.879	*Pineus coloradensis* on *Picea engelmannii*	1	1
23	Candle Lake	Saskatchewan	53.850	−104.620	*Pineus coloradensis* on *Pinus banksiana*	0	1
24	Birch Lakes State Forest	Minnesota	45.764	−94.768	*Pineus strobi* on *Pinus strobus*	13	15
25	Cross Lake	Minnesota	46.656	−94.069	*Pineus strobi* on *Pinus strobus*	1	2
26	Grand Rapids	Minnesota	47.252	−93.510	*Pineus strobi* on *Pinus strobus*	0	1
27	Chattahoochee National Forest	Georgia	34.743	−83.840	*Pineus strobi* on *Pinus strobus*	16	25
28	Celo	North Carolina	35.824	−82.187	*Adelges tsugae* on *Tsuga canadensis*	2	2
29	Mt. Rogers	Virginia	36.772	−81.175	*Pineus strobi* on *Pinus strobus*	4	5
30	Jefferson National Forest	Virginia	37.128	−80.864	*Pineus strobi* on *Pinus strobus*	12	17
31	Boalsburg	Pennsylvania	40.774	−77.812	*Pineus strobi* on *Pinus strobus*	1	2
32	Rochester	New York	43.128	−77.612	*Pineus strobi* on *Pinus strobus*	1	1
33	Ithaca	New York	42.445	−76.476	*Pineus strobi* on *Pinus strobus* and *Adelges tsugae* on *Tsuga canadensis*[a]	2	4
34	Overlook Mountain	New York	42.076	−74.126	*Pineus strobi* on *Pinus strobus*	2	2
35	Bridgewater	Connecticut	41.470	−73.319	*Pineus strobi* on *Pinus strobus*	1	1
36	Hartford	Connecticut	41.727	−72.693	*Pineus strobi* on *Pinus strobus*	6	6
37	Amherst	Massachusetts	42.451	−72.529	*Pineus strobi* on *Pinus strobus*	6	7
38	Hillsborough	New Hampshire	43.014	−71.928	*Pineus strobi* on *Pinus strobus*	9	12
39	Worcester	Massachusetts	42.279	−71.773	*Pineus strobi* on *Pinus strobus*	2	3
40	Pawtuckaway State Park	Massachusetts	43.087	−71.157	*Pineus strobi* on *Pinus strobus*	1	1
41	Arnold Arboretum	Massachusetts	42.299	−71.127	*Pineus boerneri* on *Pinus henryi*	0	1

[a]Three samples collected from *P. strobi* and 1 from *A. tsugae*.

Table 2. *Leucotaraxis piniperda* sampling locations. Location number refers to those indicated in Fig. 1B

Location number	Location name	State/province	Latitude	Longitude	Prey and host	*N* microsatellite	*N* COI
1	Mount Washington	British Columbia	49.746	−125.320	*Adelges tsugae* on *Tsuga mertensiana*	3	4
2	Olympia	Washington	47.238	−122.672	*Adelges tsugae* on *Tsuga heterophylla*	71	92
3	San Juan Islands	Washington	48.641	−122.787	*Adelges tsugae* on *Tsuga heterophylla*	16	0
4	Whidbey Island	Washington	48.133	−122.604	*Adelges tsugae* on *Tsuga heterophylla*	26	36
5	Vancouver	Washington	45.703	−122.671	*Adelges tsugae* on *Tsuga heterophylla*	17	18
6	Seattle	Washington	47.688	−122.415	*Adelges tsugae* on *Tsuga heterophylla*	0	11
7	West Valley	Washington	46.498	−121.184	*Pineus coloradensis* on *Pinus albicaulis*	2	2
8	Surrey Lake	British Columbia	50.380	−120.578	*Adelges piceae* on *Abies lasiocarpa*	0	1
9	Eldorado National Forest	California	38.915	−120.39	*Pineus coloradensis* on *Pinus* sp.	0	1
10	Salt Lake	Utah	40.713	−111.725	*Adelges piceae* on *Abies lasiocarpa*	6	6
11	Zimmerman Lake	Colorado	40.540	−105.879	*Pineus coloradensis* on *Picea engelmannii*	0	1
12	Smeaton	Saskatchewan	53.618	−104.741	*Pineus pineoides* on *Picea* sp.	0	1
13	Bemidji	Minnesota	47.470	−94.879	*Pineus* sp. on *Picea glauca*	1	1
14	Clever	Missouri	37.015	−93.363	*Pineus strobi* on *Pinus strobus*	41	49
15	Lake Ann	Michigan	44.744	−85.913	*Pineus strobi* on *Pinus strobus*	39	52
16	Blue Rock State Forest	Ohio	39.736	−81.780	*Pineus strobi* on *Pinus strobus*	5	6
17	Blacksburg	Virginia	37.135	−80.713	*Pineus strobi* on *Pinus strobus*	9	14
18	Mount Morris	Pennsylvania	39.773	−80.148	*Pineus strobi* on *Pinus strobus*	36	36
19	Shepherdstown	West Virginia	39.486	−77.805	*Pineus strobi* on *Pinus strobus*	3	3
20	Rochester	New York	43.194	−77.530	*Pineus strobi* on *Pinus strobus*	11	18
21	McCarthy Hill State Forest	New York	42.098	−77.194	*Pineus strobi* on *Pinus strobus*	8	9
22	Ithaca	New York	42.545	−76.674	*Pineus strobi* on *Pinus strobus*	55	74
23	Overlook Mountain	New York	42.076	−74.126	*Pineus strobi* on *Pinus strobus*	13	12
24	Cylburn Arboretum	Maryland	39.351	-76.652	*Pineus boerneri* on *Pinus densiflora*	7	7
25	Hamden	Connecticut	41.405	−72.926	*Pineus strobi* on *Pinus strobus*	1	9
26	Hartford	Connecticut	41.686	−72.798	*Pineus strobi* on *Pinus strobus*	23	23
27	Amherst	Massachusetts	42.403	−72.529	*Pineus strobi* on *Pinus strobus*	3	6
28	Arnold Arboretum	Massachusetts	42.279	−71.773	*Pineus strobi* on *Pinus strobus*	0	1

adelgid-infested conifer hosts under a dissecting microscope. In some cases, paint brushes were used to sweep adelgids and immature silver flies from bark into gallon-sized plastic bags, which were then transferred to glass jars with 95% ethanol and stored at 4°C before examination under the microscope. Adult silver flies were reared out of adelgid-infested host plant material or colony brushings. Immature silver flies were identified using DNA barcode clustering (described below) and adults were identified using morphological characters described in Gaimari and Havill (2021). Adelgid prey were identified to species morphologically using the keys and characters described

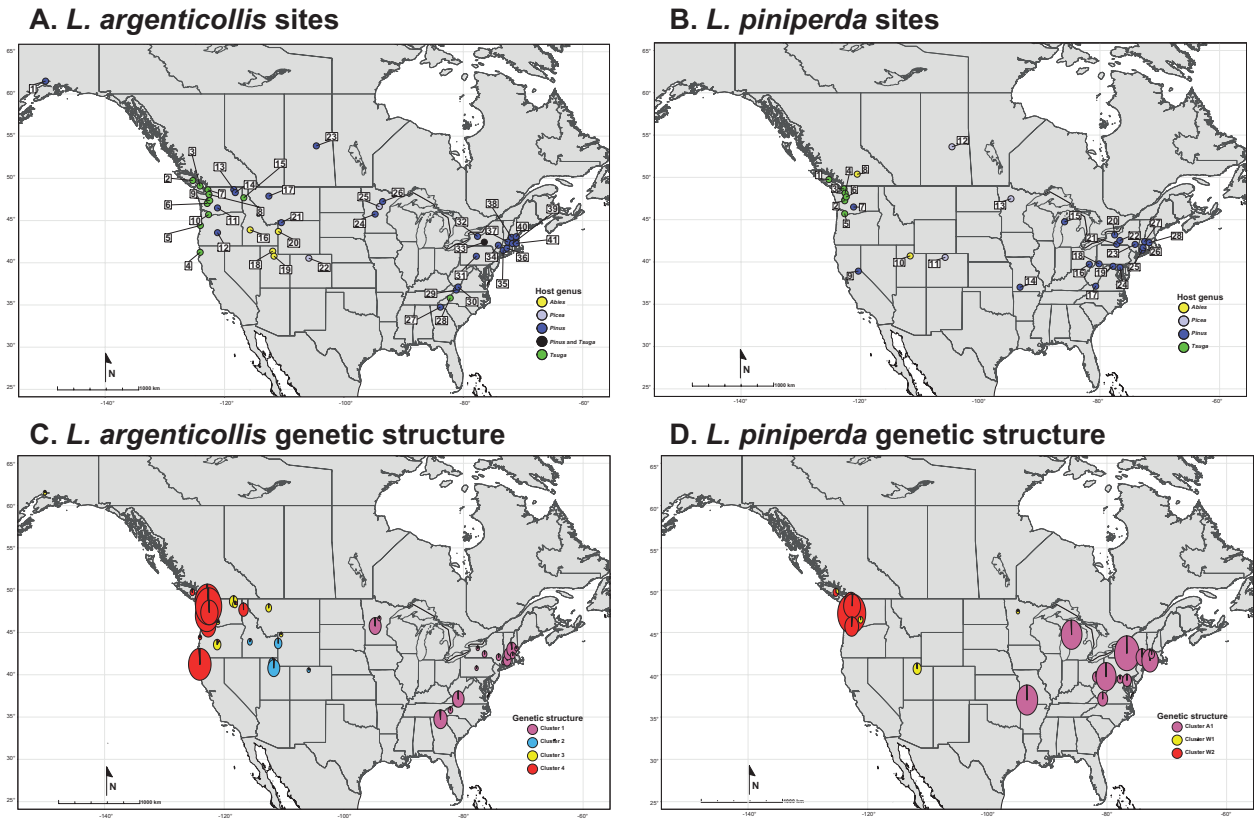

Fig. 1. Maps showing sample sites indicating the host plant genus of the adelgid prey from which flies were collected for: A) *L. argenticollis,* and B) *L. piniperda;* and pie charts showing population genetic structure for: C) *L. argenticollis,* and D) *L. piniperda.* Structure results correspond to the plots shown in Figs. 3 and 4.

in Blackman and Eastop (1994) and/or with DNA barcodes generated using the methods described below.

DNA was nondestructively extracted either using the DNA IQ Extraction Kit (Promega Corp., Madison, WI USA), the Mag-Bind DNA Extraction Kit (Omega Bio-Tek, Norcross, GA), or the MagMAX DNA Multi-Sample Ultra Kit (Applied Biosystems, Waltham, MA) on a KingFisher Flex instrument (Thermo Fisher, Waltham, MA). For all silver fly life stages and adelgids, each individual was pierced with a clean flame-sterilized insect pin or fine-tipped scalpel, and the body was removed after digestion in tissue lysis buffer overnight with Proteinase K. Immature silver fly cuticles and adelgids were slide mounted in Canada balsam. Vouchers for silver flies and adelgid prey were deposited in the Peabody Museum of Natural History, Yale University, New Haven, Connecticut, USA (YPM), the Canadian National Collection of Insects, Arachnids and Nematodes (CNC), the Cornell University Insect Collection, Ithaca, New York, USA (CUIC), or the California State Collection of Arthropods, Sacramento, California, USA (CSCA) (Supplementary Files S1 and S2).

Microsatellite Development

For microsatellite discovery and primer design, we obtained whole genome shotgun sequences from DNA extracts of *L. argenticollis* and *L. piniperda* individuals collected from each geographic region (i.e., eastern and western North America). Following Havill et al. (2018), western samples are those collected west of the Great Plains and eastern samples are those collected east of the Great Plains. To provide sufficient DNA quantities for library preparation, multiple extracts from each species by region combination were pooled: (i) *L. argenticollis* east, 10 individuals total from Minnesota, Pennsylvania, and Virginia; (ii) *L. argenticollis* west, 40 individuals from Washington; (iii) *L. piniperda* east, 22 individuals total from Connecticut, New York, and Pennsylvania; (iv) *L. piniperda* west, 40 individuals from Washington. After pooling, DNA was precipitated in sodium acetate and ethanol with GlycoBlue (Thermo Fisher), then resuspended in water. Equal amounts of DNA from each group of flies were used to prepare separate 350-bp paired-end genomic libraries using the TruSeq Nano DNA Low Throughput Library Prep Kit (Illumina, San Diego, CA). Libraries were barcoded, pooled, and sequenced on a single Illumina MiSeq flow cell at the Cornell University Biotechnology Resource Center.

Trimmomatic v.0.36 (Bolger et al. 2014) was used to remove adapters and trim poor-quality bases using a sliding window of four bases and minimum average quality of 15. Paired ends were then assembled using Pear v.0.9.11 (Zhang et al. 2014). Assembled reads are available from NCBI BioProject PRJNA852237. Microsatellite discovery and primer design using assembled reads from each group was performed using QDD 3.1.2 (Meglécz et al. 2014) with default parameters. Resulting loci were filtered to select those that contained pure microsatellites with at least six uninterrupted repeats and fewer than 10 reads in consensus sequences (to avoid duplicated loci). The resulting loci were screened to identify those that were present in both the eastern and western group for each species by using the "map to reference" command in Geneious 9.0.5 (Kearse et al. 2012). Loci with matching reads in both the eastern and western groups were examined to ensure the primers designed by QDD matched with two or fewer mismatches. Primers with mismatches were modified with appropriate degenerate nucleotides.

The selected loci were tested for amplification success using a test panel of four individuals from each group: (i) eastern *L. argenticollis*, two individuals from Minnesota and two from Virginia; (ii) western *L. argenticollis*, three individuals from Washington and one from Montana; (iii) eastern *L. piniperda*, four individuals from New York; (iv) western *L. piniperda*, four individuals from Washington. For this initial evaluation, forward primers were modified with a 5′ M13 tail (TCCCAGTCACGACGT) for incorporation of an M13 oligo labelled with 6-FAM (Boutin-Ganache et al. 2001). For these, and all subsequent microsatellite PCRs, reverse primers were modified with a 5' GTTT "pigtail" sequence (Brownstein et al. 1996) to reduce stutter. PCRs were performed in 10 µl volumes containing: 1X PCR Buffer, 1.0 µl dNTPs (10 mM each; New England Biolabs), 0.8 µl MgCl2 (25 mM), 0.025 µl of forward primer (10 mM), 0.25 µl of reverse primer (10 mM), 0.05 µl of 6-FAM labeled M13 primer (100 mM; Thermo Fisher), 0.10 µl Go Taq G2 DNA polymerase and 1.0 µl template DNA. For these, and all subsequent microsatellite PCRs, a touchdown thermocycler program was used: 95°C for 2 min (1 cycle), 95°C for 45 s, 61°C decreasing 2°C for each cycle for 30 s, and 72°C for 45 s (5 cycles), 95°C for 45 s, 51°C for 30 s, and 72°C for 45 s (30 cycles), and final extension of 72°C for 2 min (1 cycle). For all microsatellite analyses, PCR products were combined with a LIZ 500 internal size standard (Gel Company; San Francisco, CA, USA) and run on an ABI 3730 sequencer (Thermo Fisher) at the Yale University DNA Analysis Facility on Science Hill. Alleles were scored using the microsatellite plugin in Geneious.

Loci that yielded amplification products with strong peaks for at least seven of the eight test samples per species were modified with fluorescent forward primers labeled with 6-FAM (Integrated DNA Technologies, Coralville, Iowa), VIC, PET, or NED (Thermo Fisher). For each species, these primer pairs were organized into multiplex groups of three or four loci based on amplicon length and dye color for separation during analysis. For each multiplex group designed for each species, individuals were genotyped using 10 µl reactions containing 1X Type-it PCR Master Mix (Qiagen, Hilden, Germany), 0.2 µl of each dye-labeled forward primer (10 mM) and 0.2 µl pf each reverse primer (10 mM) per multiplex group, and 1 µl of DNA template. The strength of the peaks and presence of overlapping scores were analyzed for each group to identify optimal combinations. The final optimized multiplex groups are listed in Supplementary Tables S1 and S2.

Population Genetic Analyses

Collection sites were grouped into populations if they were within 40 km of each other. For each population with at least 10 individuals, Arlequin 3.5 (Excoffier and Lischer 2010) was used to calculate alleles per locus, observed and expected heterozygosity, test for differentiation among populations measured by F_{ST} using the infinite-allele model and 1,000 permutations, and test for departures from Hardy-Weinberg equilibrium and linkage equilibrium. False positives due to multiple comparisons were accounted for using the method of Benjamini and Hochberg (1995) with a false discovery rate of 0.05.

Individual-based isolation by distance was assessed separately for eastern and western individuals for each species with Mantel tests with 999 randomizations implemented in Genalex v6.5 (Peakall and Smouse 2012). Genetic distance among individuals was measured using Φ_{PT} calculated via analysis of molecular variance, a metric that is analogous to F_{ST} (Peakall et al. 1995). Euclidean geographic distances were calculated from collection site coordinates. One *L. piniperda* sample collected from Minnesota (sample 09-123-01) was excluded from the isolation by distance analysis as it clustered with western individuals of this species (see Results, below) and was the only unexplained geographic outlier.

Population genetic structure was estimated using the Bayesian clustering method implemented in Structure 2.3.4 (Pritchard et al. 2000). All Structure runs used the correlated allele frequency and admixture ancestry models with 50,000 burn-in Markov chain Monte Carlo generations, and 200,000 sample generations. For each species, 20 independent Structure runs were completed for each value of *K* from 1 to 8, and the value of *K* that demonstrated biologically meaningful groups were chosen by examining plots of *K* versus mean Ln Pr(*K*|*X*) (Pritchard et al. 2000), and *K* versus Δ*K* (Evanno et al. 2005), as implemented in Structure Harvester v0.6.94 (Earl and vonHoldt 2012), as well as examining the consensus plots for each distinct mode of Structure runs for each value of *K*, compiled using Clumpak (Kopelman et al. 2015). In addition, separate runs were completed for eastern and western samples for each species for each value of *K* from 1 to 6 to examine the hierarchical regional population structure.

DNA Barcoding and Sequence Analysis

For *Leucotaraxis* and adelgid specimens, the standard DNA barcoding region of the 5′ end of the mitochondrial COI gene was amplified in 25 ul reactions containing 1X PCR Buffer, 2.4 µl dNTPs (10 mM each; New England Biolabs, Ipswich, MA), 3.7 µl $MgCl_2$ (25 mM), 1.0 µl of each primer LepF1 and LepR1 (10 mM; Hebert et al. 2004), 0.2 µl Go Taq G2 DNA polymerase Kit (Promega), and 3 or 5 µl template DNA. The thermocycler profile consisted of 1 cycle at 95°C for 2 min, then 36 cycles at 95°C for 30 s, 46°C for 30 s, and 72°C for 1 min (30 cycles), and a final extension of 72°C for 2 min. PCR products were sequenced at the Biotechnology Resource Center at Cornell University or at Yale University's DNA Analysis Facility on Science Hill or the Yale University Keck DNA Sequencing Facility on an ABI 3730 sequencer (Thermo Fisher). Chromatograms were examined and edited using Geneious. For the identification of immature flies, DNA barcode sequences were compared to sequences from adults determined using morphology. All novel sequences generated in this study were deposited in GenBank under the accession numbers listed in Supplementary Files S1 and S2. The relationships among *Leucotaraxis* DNA barcode haplotypes were estimated using statistical parsimony networks (Templeton et al. 1992) implemented in TCS 1.21 (Clement et al. 2000) with a 95% connection limit.

Results

Sample Collection

A total of 467 *L. argenticollis* and 533 *L. piniperda* individuals were used in this study (Tables 1 and 2, Supplementary Files S1 and S2). The *L. argenticollis* sample consisted of 4 eggs, 224 larvae, 38 puparia, and 201 adults, and the *L. piniperda* sample consisted of 247 larvae, 142 puparia, and 145 adults.

Leucotaraxis argenticollis was collected in the west from four different adelgid species: (i) *Adelges tsugae* on *Tsuga heterophylla* (Raf.) Sarg. (*N* = 305) and *T. mertensiana* (Bong.) Carr. (*N* = 5); (ii) *Pineus coloradensis* (Gillette) on *Pinus contorta* Douglas (*N* = 14), *Pinus albicaulis* Engelm. (*N* = 1), *Pinus ponderosa* Douglas ex C. Lawson (*N* = 2), *Pinus flexilis* E. James (*N* = 3), *Pinus banksiana* Lamb. (*N* = 1), and *Picea engelmannii* Parry ex Engelm. (*N* = 1); (iii) *Adelges piceae* (Ratzeburg) on *Abies lasiocarpa* (Hooker) Nuttall (*N* = 21); and (iv) *Pineus abietinus* Underwood & Balch on *Abies lasiocarpa* (*N* = 6) (Supplementary Table S1). *Leucotaraxis*

argenticollis was collected in the east from four different adelgid species: (i) *Pineus strobi* (Hartig) on *Pinus strobus* L. (*N* = 102); (ii) *Pineus boerneri* Annand on *Pinus henryi* Mast. (*N* = 1); (iii) *Pineus pinifoliae* (Fitch) on *Pinus strobus* (*N* = 1); and (iv) *Adelges tsugae* on *Tsuga canadensis* (L.) Carrière (*N* = 3) (Supplementary Table S1).

Leucotaraxis piniperda was collected in the west from four different adelgid species: (i) *Adelges tsugae* on *Tsuga heterophylla* (*N* = 185) and *T. mertensiana* (*N* = 4); (ii) *Pineus coloradensis* on *Pinus albicaulis* (*N* = 2), *Pinus* sp. (*N* = 1), and *Picea engelmannii* (*N* = 1); (iii) *Pineus pineoides* (Cholodkovsky) on *Picea* sp. (*N* = 1); and (iv) *Adelges piceae* on *Abies lasiocarpa* (*N* = 7) (Supplementary Table S1). *Leucotaraxis piniperda* was collected in the east from three known and one unknown adelgid species: (i) *Pineus strobi* on *Pinus strobus* (*N* = 324); (ii) *Pineus boerneri* on *Pinus densiflora* Siebold & Zucc. (*N* = 7); (iii) *Pineus pini* (Goeze) on *Pinus sylvestris* L. (*N* = 1); and (iv) *Pineus* sp. on *Picea glauca* (Moench) Voss (*N* = 1) (Supplementary Table S1).

Microsatellite Development

After trimming and assembling the paired Illumina sequence reads, there were 3,138,887 sequences for eastern *L. argenticollis*, 3,265,405, for western *L. argenticollis*, 1,778,452 for eastern *L. piniperda*, and 7,907,215 for western *L. piniperda*. The median sequence length was 247 base pairs for all groups. Of these reads, 97,334 contained microsatellite repeats for eastern *L. argenticollis*, 96,548 for western *L. argenticollis*, 6,954 for eastern *L. piniperda*, and 229,764 for western *L. piniperda*. After primer design and filtering with QDD, there were 1,320 loci available for eastern *L. argenticollis*, 1,709 for western *L. argenticollis*, 338 for eastern *L. piniperda*, and 4,893 for western *L. piniperda*. For *L. argenticollis*, there were 118 loci with reads in both the eastern and western groups, and for 31 of these, the primers designed by QDD aligned to both reads with two or fewer mismatches. For *L. piniperda*, there were 86 loci with reads in both the eastern and western groups, and for 31 of these, the primers designed by QDD aligned to both reads with two or fewer mismatches. For each species, preliminary testing of the candidate loci indicated that there was consistent amplification and that the loci were polymorphic for 15 loci for *L. argenticollis* and 16 loci for *L. piniperda*. Primer sequences, target species, and repeat motifs are shown in Supplementary Tables S1 and S2.

Population Genetic Analyses

Microsatellite genotypes were generated for 396 individuals for each *Leucotaraxis* species. For the sites with 10 or more individuals sampled for *L. argenticollis* (*N* = 10) and *L. piniperda* (*N* = 11) (Tables 3 and 4), there were just two pairs of loci that were found to have significant linkage out of 1,230 possible pairs in *L. piniperda* (population 17, loci LP08 and LP29, plus locus LP08 and LP31), and zero out of 1050 possible pairs in *L. argenticollis*.

For *L. argenticollis*, pairwise F_{ST} values (Table 3) ranged from 0.2398 to 0.4065 between eastern and western sites, from 0.0122 to 0.0210 between eastern sites collected from the same prey species (*Pineus strobi* on *Pinus strobus*), and from 0.0004 to 0.0145 between western sites collected from the same prey (*Adelges tsugae* on *Tsuga heterophylla*). In contrast to these low values, the Salt Lake, Utah site, which was the only site with 10 or more individuals collected from *Adelges piceae* on *Abies lasiocarpa*, had uniformly high F_{ST} values ranging from 0.3910 to 0.4253 relative to both eastern and western sites collected on other adelgid prey species indicating that this group was more diverged than the others. It was not possible to compare pairwise F_{ST} values between sets of *L. argenticollis* collected on the other adelgid prey species because of low sample sizes. For *L. piniperda* (Table 4), all sites with 10 or more sampled individuals in the east (*N* = 6) had flies collected from *Pineus strobi* on *Pinus strobus*, and in the west all sites with 10 or more sampled individuals (*N* = 4) had flies collected from *Adelges tsugae* on *Tsuga heterophylla*. Pairwise F_{ST} values between eastern and western sites ranged from 0.2661 to 0.3347, F_{ST} between western sites ranged from 0.0042 to 0.0216, and F_{ST} between eastern sites ranged from 0.0000 to 0.0168. It was not possible to compare pairwise F_{ST} values between sets of *L. piniperda* collected on the other adelgid prey species because of low sample sizes.

There was a significant linear relationship between individual genetic and geographic distances for western *L. argenticollis* ($P = 0.001$, $R^2 = 0.3171$) and western *L. piniperda* ($P = 0.001$, $R^2 = 0.1687$), but not for eastern *L. argenticollis* ($P = 0.100$, $R^2 = 0.0025$) or eastern *L. piniperda* ($P = 0.383$, $R^2 = 0.00004$) (Fig. 2). The genetic distance between flies collected from different hosts was uniformly high (Fig. 2, orange points), regardless of the geographic distance between them.

Structure analyses of *L. argenticollis* microsatellite genotypes suggested a biologically sensible population differentiation of $K = 4$ clusters associated with geography and adelgid prey identity (Figs. 1 and 3). The plot of K vs. Ln Pr($X|K$) started to plateau at $K = 2$ (Supplementary Fig. S1), and the plot of K vs. ΔK (Supplementary Fig. S2) also indicated that $K = 2$ clusters separating eastern vs. western samples was the optimal clustering pattern. The consensus plots for each value of K indicated distinct clustering patterns from $K = 2$ to $K = 4$ and no additional distinct clusters thereafter (Supplementary Fig. S3). For the nested analysis with only western *L. argenticollis*, the plot of K vs. Ln Pr($X|K$) roughly plateaued at $K = 2$ or $K = 3$ (Supplementary Fig. S4), and the plot of K vs. ΔK suggested that $K = 2$ is optimal (Supplementary Fig. S5). Examination of the consensus plots for each value of K from 1 to 6 indicated distinct clustering patterns for $K = 2$ and $K = 3$ and no additional distinct clusters thereafter (Supplementary Fig. S6). The three western clusters are associated with adelgid prey species and their host plants (Figs. 1 and 3). In contrast, hierarchical analysis of eastern *L. argenticollis* did not reveal any nested clusters, suggesting K = 1 for this group (Supplementary Figs. S7–S9). Since the hierarchical anayses produced the same clustering pattern as the analysis of the full data set with $K = 4$, for simplicity we chose to display the results from the latter in Figs. 1 and 3.

The *L. argenticollis* clusters were associated with geography, prey species, and their host plants (Figs. 1 and 3). Cluster 1 (individuals with probability of assignment > 0.80) consisted of all eastern samples which were collected from *Pineus strobi* on *Pinus strobus* (*N* = 76), and *Adelges tsugae* on *Tsuga canadensis* (*N* = 3). Cluster 2 (probability of assignment > 0.80) consisted of flies collected from *Adelges piceae* on *Abies lasiocarpa* (*N* = 20), *Pineus abietinus* on *Abies lasiocarpa* (*N* = 5), and *Pineus coloradensis* on *Picea engelmannii* (*N* = 1) (Fig. 1A, site 22). Cluster 3 (probability of assignment > 0.80) consisted of silver flies collected from *Pineus coloradensis* on *Pinus albicaulis* (*N* = 1), *Pinus contorta* (*N* = 12), *Pinus flexilis* (*N* = 3), and *Pinus ponderosa* (*N* = 1). Cluster 4 (probability of assignment > 0.80) consisted of flies collected from *Adelges tsugae* on *Tsuga heterophylla* (*N* = 269), and *T. mertensiana* (*N* = 3). Two individuals collected from *Pineus coloradensis* on *Pinus ponderosa* had split assignments to Clusters 3 and 4 with probabilities of 0.59–0.40, and 0.46–0.54, respectively.

Hierarchical Structure analyses of *L. piniperda* microsatellite genotypes suggested a biologically sensible population differentiation with hierarchical clustering of $K = 2$ clusters for analysis of

Table 3. Pairwise population differentiation (F_{ST}) calculated using 15 microsatellite loci for *Leucotaraxis argenticollis* populations with 10 or more individuals. Asterisks denote values that are significantly different from zero using the multiple comparison method of Benjamini and Hochberg (1995) with a false discovery rate of 0.05. Population numbers and names correspond to those in Table 1 and Fig. 1

	4. Trinidad, California	6. Olympia, Washington	7. San Juan Islands, Washington	8. Whidbey Island, Washington	9. Tacoma, Washington	10. Vancouver, Washington	19. Salt Lake, Utah	24. Birch Lakes State Forest, Minnesota	25. Chattahoochee National Forest, Georgia
6. Olympia, Washington	0.0145*								
7. San Juan Islands, Washington	0.0089*	0.0052*							
8. Whidbey Island, Washington	0.0060*	0.0018	0.0039						
9. Tacoma, Washington	0.0043	0.0029	0.0004	0.0010					
10. Vancouver, Washington	0.0111*	0.0050	0.0046	0.0031	0.0013				
19. Salt Lake, Utah	0.4120*	0.3964*	0.4030*	0.4001*	0.4253*	0.4158*			
24. Birch Lakes State Forest, Minnesota	0.2703*	0.2586*	0.2668*	0.2662*	0.2674*	0.2578*	0.4053*		
25. Chattahoochee National Forest, Georgia	0.2569*	0.2398*	0.2467*	0.2470*	0.2486*	0.2433*	0.3910*	0.0132*	
30. Jefferson National Forest, Virginia	0.2769*	0.2645*	0.2722*	0.2705*	0.2731*	0.2665*	0.4065*	0.0122*	0.0210*

all individuals that separated eastern versus western individuals, with nested $K = 2$ clusters for western silver flies associated with geography and adelgid prey identity, and no additional nested clustering for eastern silver flies (Fig. 4). For all individuals, the plot of K vs. Ln Pr($X|K$) started to plateau at $K = 2$ (Supplementary Fig. S10), and the plot of K vs. ΔK (Supplementary Fig. S11) also suggested that $K = 2$ clusters were optimal. The Structure plot for $K = 2$ separated eastern versus western individuals, with some mixed assignments of western individuals collected from prey other than *Adelges tsugae* (Fig. 4A). Examination of the consensus plots for each value of K from 1 to 6 indicated a distinct clustering pattern for $K = 2$ and no additional distinct clusters thereafter (Supplementary Fig. S12). For the nested analysis with only western *L. piniperda*, the plot of K vs. Ln Pr($X|K$) increased rapidly from $K = 1$ to $K = 2$, then rose again to $K = 3$, then sloped downwards from $K = 4$ to 6 (Supplementary Fig. S13). The plot of K vs. ΔK suggested that $K = 2$ is optimal, but did not drop to near zero until $K = 4$ (Supplementary Fig. S14). Examination of the consensus plots for each value of K from 1 to 6 indicated distinct clustering patterns for $K = 2$ and no additional distinct clusters thereafter (Supplementary Fig. S15). In contrast, hierarchical analysis of eastern *L. piniperda* did not reveal any additional clustering, suggesting $K = 1$ for this group (Supplementary Figs. S16–S18).

Similar to *L. argenticollis*, the *L. piniperda* clusters were associated with geography, prey species, and their host plants (Figs. 1 and 4). From the analysis of all individuals, Cluster A1 (individuals with probability of assignment > 0.80) consisted of all eastern silver flies collected from *Pineus strobi* on *Pinus strobus* ($N = 243$), *Pineus boerneri* on *Pinus densiflora* ($N = 7$), *Pineus pinifoliae* on *Pinus strobus* ($N = 3$), and *Pineus pini* on *Pinus sylvestris* ($N = 1$). Cluster A2 (probability of assignment > 0.80) consisted of all western silver flies collected on *Adelges tsugae* on *Tsuga heterophylla* ($N = 130$), and *T. mertensiana* ($N = 3$), and 1 individual collected on *Adelges piceae* on *Abies lasiocarpa*. Additional individuals with mixed assignments (probability of assignment < 0.80) were collected from *Adelges piceae* on *Abies lasiocarpa* ($N = 5$), *Pineus coloradensis* on *Pinus albicaulis* ($N = 2$), and *Pineus* sp. on *Picea glauca* ($N = 1$), and *Adelges tsugae* on *Tsuga mertensiana* ($N = 1$). When western samples were analyzed separately, Cluster W1 (probability of assignment > 0.80) consisted of silver flies collected from *Adelges piceae* on *Abies lasiocarpa* ($N = 6$), *Pineus coloradensis* on *Pinus albicaulis* ($N = 2$), *Pineus* sp. on *Picea glauca* ($N = 1$), and *Adelges tsugae* on *Tsuga mertensiana* ($N = 1$), and Cluster W2 (probability of assignment > 0.80) consisted of flies collected from *Adelges tsugae* on *Tsuga heterophylla* ($N = 130$) and *Tsuga mertensiana* ($N = 2$). One individual from *Adelges tsugae* on *Tsuga heterophylla* had a lower probability of assignment to this cluster of 0.7208.

DNA Barcoding and Sequence Analysis

We obtained COI DNA barcode sequences from 446 *L. argenticollis* and 493 *L. piniperda* individuals (Tables 1 and 2). Some of the sequences were previously reported in one or more of the following publications: Havill et al. (2018) ($N = 606$), Wantuch et al. (2019) ($N = 22$), Rose et al. (2020) ($N = 62$), and Gaimari and Havill (2021) ($N = 48$). The haplotype networks for *L. argenticollis* (Fig. 5) and *L. piniperda* (Fig. 6) included 65 and 86 haplotypes, respectively. Within each species there were separate clusters consisting of samples collected west versus east of the Great Plains that did not connect with a 95% connection limit

Table 4. Pairwise population differentiation (F_{ST}) calculated using 16 microsatellite loci for *Leucotaraxis piniperda* populations with 10 or more individuals. Asterisks denote values that are significantly different from zero using the multiple comparison method of Benjamini and Hochberg (1995) with a false discovery rate of 0.05. Population numbers and names correspond to those in Table 2 and Fig. 1

	2. Olympia, Washington	3. San Juan Islands, Washington	4. Whidbey Island, Washington	5. Vancouver, Washington	14. Clever, Missouri	15. Lake Ann, Michigan	18. Mount Morris, Pennsylvania	20. Rochester, New York	22. Ithaca, New York	23. Overlook Mountain, New York
3. San Juan Islands, Washington	0.0110									
4. Whidbey Island, Washington	0.0042	0.0136								
5. Vancouver, Washington	0.0216*	0.0086	0.0184							
14. Clever, Missouri	0.3167*	0.2956*	0.2887*	0.2761*						
15. Lake Ann, Michigan	0.3113*	0.2910*	0.2845*	0.2707*	0.0066					
18. Mount Morris, Pennsylvania	0.3044*	0.2841*	0.2757*	0.2661*	0.0054	0.0000				
20. Rochester, New York	0.3344*	0.3171*	0.3076*	0.2924*	0.0105	0.0063	0.0020			
22. Ithaca, New York	0.3046*	0.2877*	0.2802*	0.2691*	0.0073	0.0061	0.0038	0.0066		
23. Overlook Mountain, New York	0.3347*	0.3235*	0.3112*	0.2978*	0.0121	0.0046	0.0067	0.0072	0.0124	
26. Hartford, Connecticut	0.3292*	0.3082*	0.3004*	0.2910*	0.0040	0.0073	0.0054	0.0168*	0.0134*	0.0127

of 11 mutations. The one exception to this pattern was a sample of *L. piniperda* collected from Minnesota (sample 09-123-01) with a mitochondrial haplotype that clustered with the western samples. The mean pairwise COI p-distance between western and eastern *L. argenticollis* was 0.0483 (min: 0.0395; max: 0.0564), and between western and eastern *L. piniperda* was 0.0526 (min: 0.0400; max: 0.0681).

In both species, the western clusters exhibited longer branches, indicative of a longer history of divergence than the eastern clusters, with a dominant haplotype with shallow connections to numerous low-frequency haplotypes. In *L. argenticollis*, most of the samples collected from adelgids on *Abies* hosts were at the end of long branch, indicating some level of structure associated with adelgid prey. There was no obvious structure associated with prey species in the *L. piniperda* haplotype network.

Discussion

Host-Associated Differentiation

Host-associated differentiation is recognized as a generator of speciation for groups of organisms that co-occur geographically but have become specialized on different hosts (Berlocher and Feder 2002, Drès and Mallet 2002, Matsubayashi et al. 2010). In insects, these associations have been shown for herbivores (e.g., Via 1999, Forbes et al. 2009, Peccoud and Simon 2010, Hood et al. 2015, Bakovic et al. 2019, Driscoe et al. 2019), and parasitoids that prefer different herbivores that specialize on different host plants (e.g., Stireman et al. 2006, Abrahamson and Blair 2008, Hood et al. 2015). Here we present less-common examples of host-associated differentiation for insect predators. Genetic analyses of *L. argenticollis* and *L. piniperda* confirmed that within both species there are allopatric east–west genetic divergences (reported previously in Havill et al. 2018, Gaimari and Havill 2021). The additional analyses reported here, using finer-scale genetic markers, show additional population differentiation associated with adelgid prey species that are specific to different host plants in western North America.

In eastern North America, where individuals of both species were collected almost exclusively from pine adelgids, there was no additional prey- or host plant-associated genetic structure (Figs. 1, 3, and 4). We should caution, however, that the lack of further population sub-division in eastern North America could be due to low samples sizes for flies collected from other prey species in the region. For Bayesian population structure analysis, uneven sample sizes

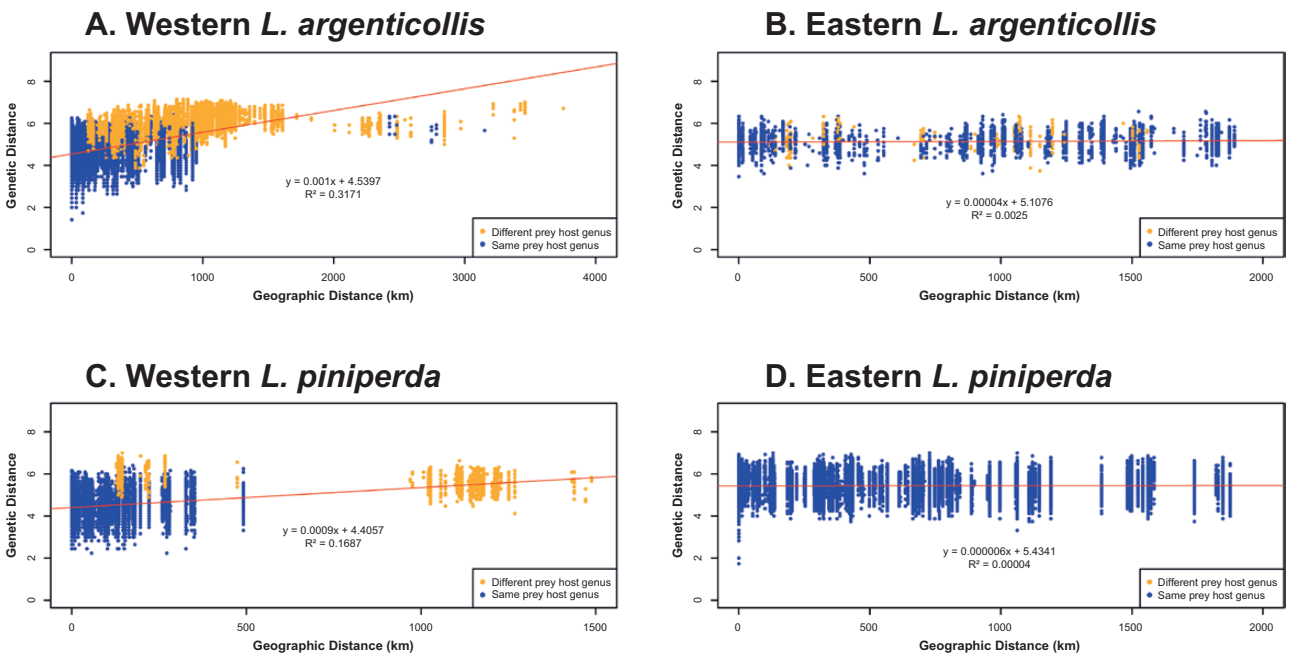

Fig. 2. Individual-based isolation by distance for eastern and western groups of both *Leucotaraxis* species. Pairwise genetic distance was calculated as linear genotypic distance and Euclidean geographic distance was calculated from collection site coordinates. Points are differentially shaded for distances between two individuals collected from the same adelgid prey host plant genus (e.g., *Tsuga*—*Tsuga*), or for two individuals collected from different adelgid prey host plant genera (e.g., *Tsuga*—*Pinus*).

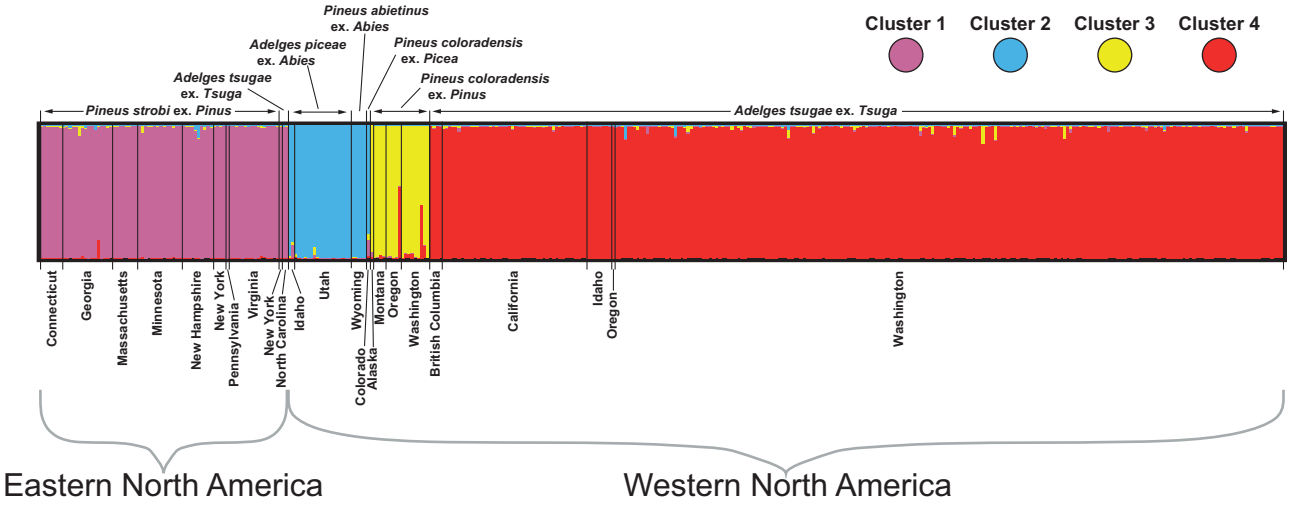

Fig. 3. STRUCTURE plot for *Leucotaraxis argenticollis* genotyped with 15 microsatellite loci. The height of each bar represents the proportion of an individual's genotype assigned to each of $K = 4$ clusters. The names of the clusters correspond to those in Fig. 1C. Vertical black lines separate groups of individuals collected in different states or provinces and on different adelgid host plant genera.

among populations can result in incorrect estimation of the number of populations and reduce the accuracy of individual assignments to populations (Puechmaille 2016, Wang 2017).

In contrast to the lack of genetic structure in the east, there were regional patterns in the west that suggest that adelgid hosts are a determinant of genetic differentiation. In particular, we saw this for *L. argenticollis* that showed a disjunct geographic pattern for genetic Cluster 4 (Fig. 1C), the lineage that feeds on *Adelges tsugae* in the west. Silver flies in this lineage were collected feeding on *A. tsugae* on *Tsuga heterophylla* and *T. mertensiana* in coastal locations (Fig. 1A, Sites 2–20), as well as in a disjunct location in northern Idaho (Fig. 1A, Site 15). Between these areas we identified individuals that fell in Cluster 3 (Fig. 1C) and were feeding on *Pineus coloradensis* on several *Pinus* host species (Fig. 1A, Sites 11–14). Additional evidence that host plant species is important for host location by *L. argenticollis* is indicated by Cluster 2 (Figs. 1 and 3) which included silver flies collected from two divergent adelgid species, *Adelges piceae* and *Pineus abietinus*, on *Abies lasiocarpa*, suggesting that flies are differentiating based on their ability to locate host plants, rather than the ability to find prey species alone.

The patterns for western *L. piniperda* are less distinct, but there is still evidence for prey-associated differentiation. The genetic structure results from analysis of all *L. piniperda* individuals (Fig. 4) might suggest that western flies collected from adelgid species

feeding on *Pinus* and *Abies* could be the product of admixture between eastern flies (Cluster A1) and western flies collected from *A. tsugae* on *Tsuga* (Cluster A2), However, since the DNA barcode sequences of all western individuals were divergent from eastern ones (Fig. 6), we suspect that this pattern emerged because the strong differentiation between eastern and western genotypes masked the hierarchical structure in the west. With hierarchical analysis of the western flies, there was clear differentiation between individuals feeding on *A. tsugae* on *Tsuga heterophylla* and *T. mertensiana* versus those feeding on other adelgids on other host plants, but unlike *L. argenticollis* there was no differentiation among individuals feeding on adelgids on *Abies* versus on *Pinus*. This may be a result of reduced specialization on adelgids other than *A. tsugae* on *Tsuga*, or could be due to the lower sample sizes of flies collected from these other adelgid species, as explained above. Adding more samples from these other adelgids could help to distinguish these alternative explanations.

These results suggest that western populations of each species are undergoing host-associated differentiation, likely reinforced through the use of specific tri-trophic cues from different host plants. These predators may be using adelgid-induced volatiles specific to different plant taxa to locate their prey (Havill and Raffa 2000). These host plant-specific patterns of prey utilization also support the hypothesis that adelgid host alternation could provide periodic temporal escape from predation (Way and Banks 1968, Havill and Foottit 2007). Indeed, the one *L. argenticollis* individual that was collected from the alternate host of *Pineus coloradensis*, *Picea engelmannii* rather than *Pinus* spp., grouped with Cluster 3 (Fig. 3), the *Abies*-associated lineage, potentially signaling a disruption of species-specific feeding in this species.

Biogeographic History

The DNA barcode haplotype networks (Figs. 5 and 6) uncovered broadly similar patterns for both *L. argenticollis* and *L. piniperda*. The western haplotype networks had deeper genetic divergences than the eastern ones, which each had a common haplotype with numerous short branches to different haplotypes. These contrasting patterns are indicative of a longer history of divergence in the west and recent diversification in the east (Bandelt et al. 1995). For both species, the increased genetic diversity in western North America was also associated with significant isolation by distance (Fig. 2), also indicating that they have had a longer historical association with the prey and their host plants in that region.

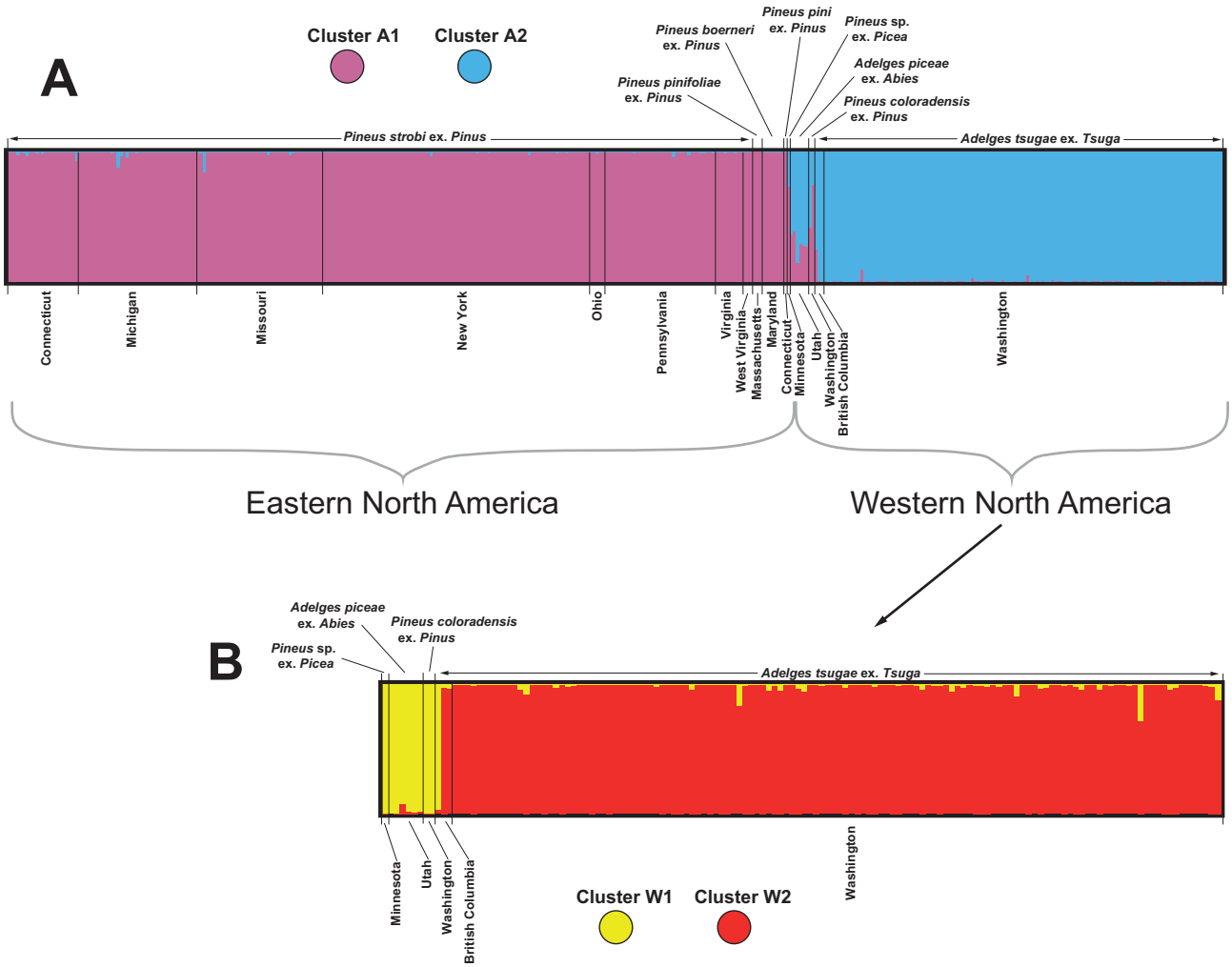

Fig. 4. STRUCTURE plot for *Leucotaraxis piniperda* genotyped with 16 microsatellite loci. The height of each bar represents the proportion of an individual's genotype assigned to: A) each of $K = 2$ clusters for analysis of all individuals, and B) each of $K = 2$ clusters for analysis of only western individuals. The names of the clusters correspond to those in Fig. 1D. Vertical black lines separate groups of individuals collected in different states or provinces and on different adelgid host plant genera.

In a phylogenetic reconstruction of Adelgidae, Havill et al. (2007) identified that adelgid species that feed on different secondary host plant genera diversified in the Late Cretaceous and Early Tertiary periods, around the time when their host plant genera were themselves differentiating. Here, our DNA barcode results suggest that the *Leucotaraxis* host shifts in the west are much more recent than the adelgid diversification on different host genera. This recency is indicated by the regional networks (Figs. 5 and 6) with only weak COI differentiation associated with feeding on different adelgids. As such, host-associated differentiation does not likely represent a strict adherence to what is generally referred to as either sequential divergence (Stireman et al. 2006, Abrahamson and Blair 2008, Forbes et al. 2009, Hood et al. 2015) or an upwards adaptive radiation cascade (Brodersen et al. 2018) in which speciation of the predator is happening at the same time, or shortly after speciation of the herbivore.

A possible historical scenario is that in North America, both *Leucotaraxis* species originated in the west, then migrated to the east where they each diverged allopatrically from their western ancestors. A recent phylogeny of the genus *Leucotaraxis* reconstructed using DNA sequence data from two nuclear genes (CAD and TPI) and mitochondrial COI DNA barcodes (Gaimari and Havill 2021) does not indicate which of the western lineages might have given rise to the eastern lineages, but the specificity of eastern silver flies on pine adelgids (species of *Pineus*) feeding on pines (species of *Pinus*) might indicate that this is the ancestral prey of both silver fly species. Note, that *L. argenticollis* also occurs in Eurasia, where it is also recorded to feed on species of *Pineus* on *Pinus* (McAlpine and Tanasijtshuk 1972, Gaimari and Havill 2021). An alternative scenario could be that eastern and western lineages are derived from a common unsampled lineage. Further research to include European specimens could provide more information about the history of prey switching in the species.

In the east, both *Leucotaraxis* species likely remained specific to pine adelgids in the absence of alternative species of native adelgids on other host genera. In contrast in the west, the silver flies diversified as they came into contact with additional adelgid prey on other host genera as they encountered them. For example, *A. tsugae* on *Tsuga* is a relatively recent addition to the western adelgid fauna, estimated to have entered western North America from east Asia before the last glacial period (ca. 57–29,000 yr ago) (Havill et al. 2016). In addition, balsam woolly adelgid, *Adelges piceae* on *Abies*, is an even more recent introduction to western North America from Europe, arriving approximately 100 yr ago (Havill et al. 2021). In this case, silver flies may have already had the ability to locate adelgids on *Abies* because of the presence of *Pineus abietinus*, the only *Pineus* species that does not feed on either *Pinus* or *Picea* (Havill and Foottit 2007). Like *A. tsugae*, *P. abietinus* is probably also a relatively recent addition to the western adelgid fauna, but this time via a recent host switch from *Pinus* to *Abies*. This is based on the close genetic similarity of *P. abietinus* to *P. similis* (Gillette), a western pine adelgid that alternates between *Picea* species and *Pinus monticola* (N.P.H, unpublished). So, a likely scenario is that both *Leucotaraxis* species originally fed on *Pineus* species on pine, and as other adelgid species on other host plants moved into contact, or experienced secondary host switches, *Leucotaraxis* were able to switch prey and evolve the ability to locate these other species more efficiently over time, leading to divergent prey-association lineages.

New Prey Records

The adelgid prey records for both of the silver fly species in this study were previously reported in Gaimari and Havill (2021) and

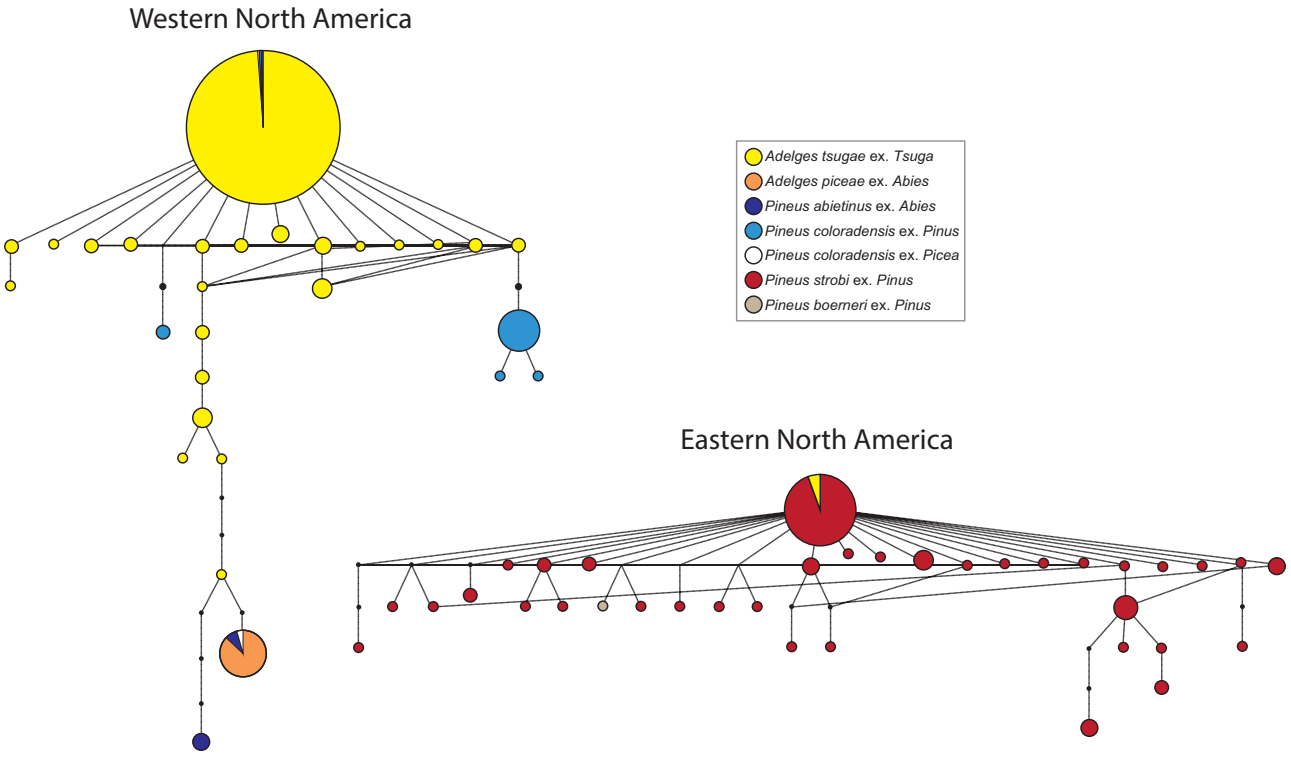

Fig. 5. Haplotype network of *Leucotaraxis argenticollis* DNA barcode sequences. The area of each pie chart is proportional to the number of samples sharing that haplotype. Small black dots represent unsampled haplotypes. Pie charts indicate the proportions of flies sampled from different host plant genera of adelgid prey.

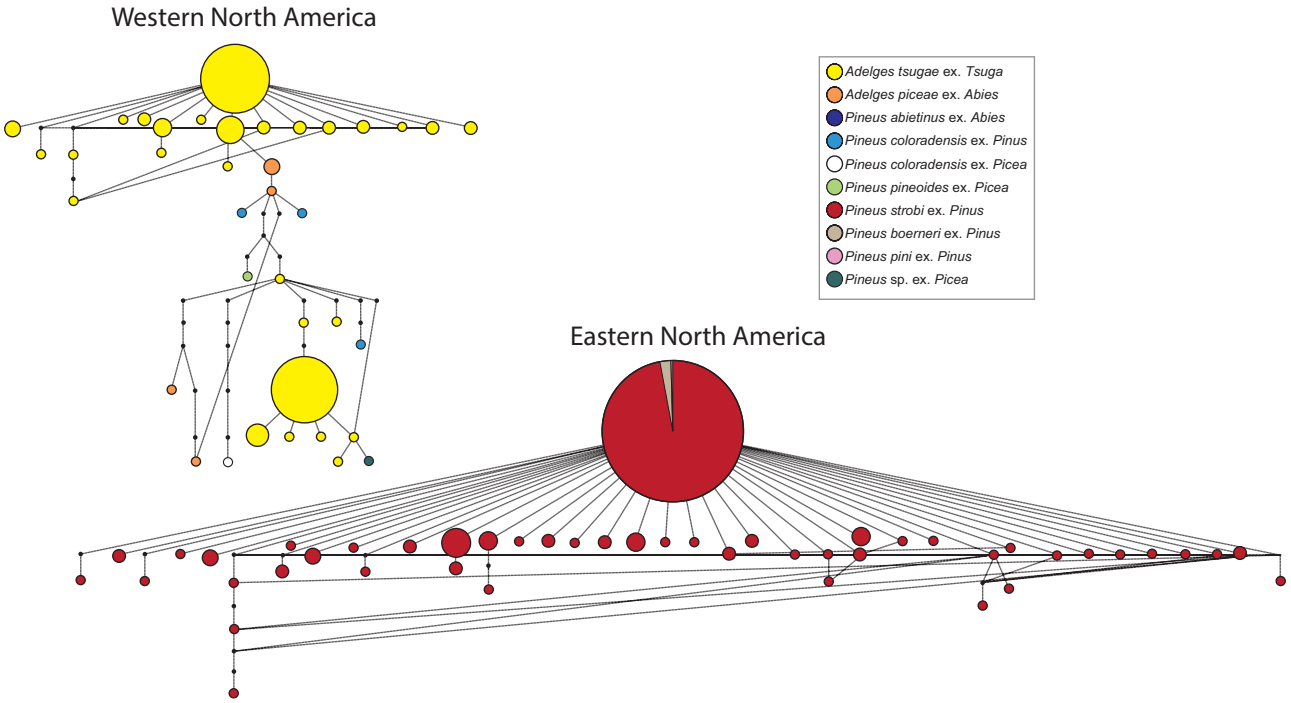

Fig. 6. Haplotype network of *Leucotaraxis piniperda* DNA barcode sequences. The area of each pie chart is proportional to the number of samples sharing that haplotype. Small black dots represent unsampled haplotypes. Pie charts indicate the proportions of flies sampled from different host plant genera of adelgid prey.

references therein, with the exception of the first confirmed records (to our knowledge) of (i) *L. argenticollis* collected from *A. tsugae* on *T. canadensis* in eastern North America; (ii) both species collected from *A. tsugae* on *T. mertensiana* in western North America; and (iii) both species collected from *Pineus pinifoliae* on *Pinus strobus* in eastern North America.

One of the three *L. argenticollis* individuals collected from *A. tsugae* on *T. canadensis* was collected in New York and two were collected in North Carolina. The individual from New York was a puparium attached to adelgid-infested *T. canadensis*. The two individuals collected in North Carolina were recovered as adults from adelgid-infested branches placed into a Lari-Leuco emergence container (Mayfield et al. 2021) designed to monitor the recovery of adelgid biological control agents. In both cases it is likely that the flies were feeding on *A. tsugae* as larvae because the infested foliage was confined in the laboratory. All three of these flies clustered, both in the microsatellite structure analysis (Fig. 3) and COI network (Fig. 5), with the genetically uniform eastern lineage that was otherwise only collected from the native pine adelgids on *Pinus strobus*. Numerous surveys for natural enemies on *A. tsugae*-infested *T. canadensis* in the eastern U.S. have been conducted that did not report the presence of silver flies (e.g., Montgomery and Lyon 1996, Mausel et al. 2008, Jones et al. 2014). Just one study, Wallace and Hain (2000), reported nine silver fly larvae feeding on *A. tsugae* in Virginia, but these were not identified to species. It is possible that these were also *L. argenticollis*, but since this prey record has not been observed elsewhere, these records likely indicate rare incidences of eastern *L. argenticollis* feeding on nonnative *A. tsugae*.

Both *Leucotaraxis* species have also not been reported previously feeding on western *A. tsugae* on *T. mertensiana*, but this could be the result of a much lower sampling effort on this tree species compared to *T. heterophylla*. One of the *L. piniperda* individuals collected from *T. mertensiana* grouped with the flies collected from adelgid species other than *A. tsugae*, perhaps suggesting some differences in host location behavior on the different *Tsuga* species.

The records of both *Leucotaraxis* species on *Pineus pinifoliae* on *Pinus strobus* are perhaps not surprising, since both silver fly species can be readily collected feeding on *Pineus strobi* (Havill et al. 2018) which uses the same host plant. *Pineus strobi* only feeds on *Pinus strobus* while *Pineus pinifoliae* alternates between *Pinus strobus* and *Picea* species in eastern North America. On *Pinus strobus*, both adelgid species have a similar habit of settling on the bark, and are sometimes found in mixed colonies (N.P.H., unpublished data). It therefore seems likely that, as with *Adelges piceae* and *Pineus abietinus* that share *Abies* hosts in the west, silver fly prey preference is based more on their ability to locate host plants, rather than adelgid prey species.

Biological Control Implications

This study provides evidence for prey-associated differentiation that can be used to improve the safety and efficacy of biological control using these species (Thompson et al. 2022). No-choice laboratory host range testing of these *Leucotaraxis* species collected from *A. tsugae* on *T. heterophylla* found that they could complete development on *A. tsugae*, *A. cooleyi* (Gillette), *A. piceae*, and *Pineus* species, but that survival was higher on *A. tsugae* than the other prey species (Grubin et al. 2011). Our findings of differentiated lineages on different adelgid prey are consistent with this study and lend further evidence for a genetic component to their prey specificity. As such, continuing to source flies from *A. tsugae* and not from other adelgid species in the west is the safer and more effective choice for release as biological control agents for *A. tsugae* in the east.

It is interesting to note that unlike in the west where both *Leucotaraxis* species were collected from the nonnative pest, *Adelges piceae*, they have not been collected in the east from this prey despite

extensive sampling (Havill et al. 2021). As discussed above, this is likely because there is a native adelgid species that also feeds on *Abies* in the west but not the east, so prey switching has not yet occurred. Despite the release of numerous natural enemies from Europe and Asia for biological control of *A. piceae*, biological control of this species has not been successful in North America (Montgomery and Havill 2014). Evaluation of the impact and further evaluation of the specificity of the *Abies* adelgid-differentiated lineage of *L. argenticollis* in the west could help determine its potential for release as a biological control agent of *A. piceae* in the east.

Potential Hybridization

In addition to the differences in host preference, there may be differences in phenology between eastern and western flies which are adapted to different climates, which could impact their potential overlap after release. However, the three records of eastern *L. argenticollis* feeding on nonnative Japanese *A. tsugae* (Fig. 3), and the one record of western *L. piniperda* feeding on *Pineus coloradensis* on *Pinus* that grouped with the *Tsuga*-associated Cluster 2 (Fig. 4) indicates that even if silver flies are collected from western *A. tsugae* in the west, the western and eastern flies will likely still encounter each other occasionally on adelgid host trees after release in the east. In a similar situation, the western predator *Laricobius nigrinus*, which is also associated with western *A. tsugae*, was released in the east as a biological control, and subsequently encountered a sibling species, *Laricobius rubidus* LeConte, which feeds primarily on *Pineus strobi* (Havill et al. 2012). The proximity of hemlock and pine within forests probably allowed for regular contact between these species, and several studies have found 2–13% hybrids feeding on *A. tsugae* while both species maintain genetic integrity on their respective preferred prey (Jones et al. 2014, Fischer et al. 2015, Mayfield et al. 2015, Wiggins et al. 2016, Jubb et al. 2020). It is unknown whether or to what extent eastern and western lineages of these two *Leucotaraxis* species will hybridize after release in the east, but the microsatellite markers and baseline genotype data reported here will allow for its tracking following the release of western flies.

Supplementary Material

Supplementary material is available at *Insect Systematics and Diversity* online.

Specimen Collection Statement

The authors attest that all legal and regulatory requirements, including export and import collection permits, have been followed for the collection of specimens from source populations at any international, national, regional, or other geographic level for all relevant field specimens collected as part of this study.

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