Am. Midl. Nat. (2017) 178:260–274

Phylogeographic Characterization of Genetic Variation in the Biological Control Agent Milfoil Weevil (Euhrychiopsis lecontei) throughout North America

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ABSTRACT—This research addresses three primary goals: (1) to ascertain the genetic diversity of natural populations of *Euhrychiopsis lecontei*, a biological control agent currently used for the management of the invasive aquatic weed, Eurasian watermilfoil; (2) to determine the presence of any potential cryptic species of *E. lecontei*; and (3) to examine the phylogeography of *E. lecontei* to determine whether geographic patterns are identifiable in the genetic diversity. Sequence data from the *cytochrome c oxidase subunit I* (COI) gene were determined for *E. lecontei* from 35 populations throughout their native range in North America. A maximum likelihood tree indicates all haplotypes of *E. lecontei* form a monophyletic group. Based on branch lengths, the tree indicates there are two major subgroupings of haplotypes. This tree structure is further supported by the haplotype network, the SAMOVA results, and the geographic barrier to gene flow which clearly demonstrates genetic and geographic structuring within the species. Multiple lines of evidence support the hypothesis all individuals sampled from a broad geographic region throughout *E. lecontei's* range represent a single species. No cryptic species were discovered in this study, although distinct geographic and genetic structuring was revealed, likely due to current geographic isolation of the sampled waterbodies, as well as the locations of historical glacial refugia.

INTRODUCTION

Phylogeography addresses the interaction between genetic variation, phylogenetic relationships, and geographic distributions (Manel *et al.*, 2003; Wang, 2010). Intraspecific phylogenetics can delineate geographic subspecies, greatly contributing to insights about regional biodiversity (Hewitt, 2004). Tools for characterizing both genetic variation and geographic patterns have continued to be developed over the past few decades (Hickerson *et al.*, 2010). As a result of these improvements, glacial refugia and their role in current distributions of both plants and animals across a range of geographic scales are becoming recognized as important contributors to biogeography and phylogeography. In North America phylogeographic studies have proposed and argued for a number of glacial refugia. In the northeastern and north central regions of the United States, the role of refugia have been examined for several species of flora (Tremblay and Schoen, 2002; Jaramillo-Correa, 2004; Godbout *et al.*, 2005) and fauna (Austin *et al.*, 2002; Lee-Yaw *et al.*, 2005; Rowe *et al.*, 2004).

Beetles (Coleoptera) are the most biologically diverse group of described insects and the Curculionidae, the "true" weevils, exceed over 60,000 described species, making up at least 17% of known Coleopterans (Bouchard *et al.*, 2009). Weevils are phytophagous insects and feed on virtually all types of plants (Oberprieler *et al.*, 2007). Within the Curculionidae, only a few subfamilies (*i.e.,* Bagoinae, Brachycerinae, Ceutorhynchinae, Curculioninae) are recognized as having species that utilize aquatic or semi-aquatic plants as hosts, and most of these species are poorly studied (Center *et al.*, 2002). Some of these aquatic weevil species have potential as biological control agents for invasive aquatic plants (*e.g.*, *Bagous* spp. feed

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on *Hydrilla*; *Neohydronomus* spp. feed on waterlettuce; *Neochetina* feed on waterhyacinth; Center *et al.*, 2002).

Milfoil weevils (*Euhrychiopsis lecontei),* whose ancestral host is *Myriophyllum sibiricum* (Northern watermilfoil), are one such potential biological control agent. These weevils are distributed throughout the northern United States and southern Canadian provinces (Creed, 1998; Newman, 2004) with all their life stages now dependent on Eurasian watermilfoil (*Myriophyllum spicatum*). Milfoil weevils overwinter on shore and do not return to the water to breed until the water temperature is approximately 15 C (Newman *et al.*, 2001). Populations of *E. lecontei* are characterized as temporally and spatially patchy (Creed and Sheldon, 1994; Tamayo *et al.*, 2000) and the exact extent of their range is unknown. For example *E. lecontei* has recently been found in California (Cline *et al.*, 2013) and is still reported as absent from Montana, Wyoming, North Dakota, and South Dakota (Creed, 1998). Accurately determining the distribution of *E. lecontei* is difficult because of the weevil's small size and its relatively low population densities, which make field sampling difficult. Therefore, perceived absence of this species in an area could simply be the result of limited sampling (*i.e.*, lack of sampling effort, inaccessibility to sites where this species actually resides, or inappropriate timing of sampling efforts). Alternatively, absence of *E. lecontei* in a certain area could reflect an actual gap in the distribution resulting from an artifact of unsuitable habitat imposed by environmental or ecological parameters. However, the range of *E. lecontei* may very well be expanding with the spread of the invasive Eurasian watermilfoil into more lakes (Jester *et al.*, 2000). Because of sampling and morphological taxonomic identification difficulties, having a relatively quick and accurate method utilizing a molecular genetic marker to determine if an individual belongs to *E. lecontei* or is a cryptic species would be a useful tool.

Research investigating the genetic structure of various organisms has utilized the number of differences in base pairs within the *cytochrome c oxidase subunit I* (COI) gene to define and assign haplotypes to different populations (Waits *et al.*, 1998; Cognato *et al.*, 2003). Haplotypes can vary by as little as one base pair and populations may contain more than one haplotype (Waits *et al.*, 1998; Cognato *et al.*, 2003). As more COI sequences are added to reference libraries (Hajibabaei *et al.*, 2007), they can function as a "global bioidentification system for animals" (Herbert *et al.*, 2003) and provide insights into complex phylogenetic and population genetic patterns. Reliance on molecular genetic markers has become an essential tool for correctly identifying weevils that are considered pests of many economically important plant species, such as the avocado stem weevil (Engstrand *et al.*, 2010), citrus root weevil (Ascunce *et al.*, 2009), and boll weevil (Barr *et al.*, 2013).

The current study aims to: (1) ascertain the genetic diversity of natural populations of *E. lecontei* by analyzing COI sequence data that have been collected for *E. lecontei* throughout their native range; (2) determine if there is cryptic speciation within the group of individuals examined in this study, using molecular markers; and (3) examine the phylogeography of *E. lecontei* to determine any geographic patterns in the genetic diversity. Geographic Information Systems (GIS) and spatial statistics are used to compare the spatial and genetic structure of *E. lecontei* populations. This information provides insight into the phylogenetics of this important biological control agent that is currently being used to manage an invasive aquatic weed, *M. spicatum* (Eurasian watermilfoil).

METHODS

Euhrychiopsis lecontei were sampled from 35 populations throughout North America (Table 1). Sample sizes ranged from one to four individuals per population for a total of 67

TABLE 1.—*Euhrychiopsis lecontei* collection locality data and sample size

Waterbody	State/province	Latitude	Longitude	Sample size (n)	Site code
Auburn Lake	Minnesota	43°35′56.55″N	88°12′23.51″W	4	ALMN
Big Bald Lake	Ontario Canada	44°34′38.15″N	78°23′31.10″W	2	BBOC
Burke Lake	Washington	47°8′0.92″N	119°55′6.10″W	1	BLWA
Cedarville Bay	Michigan	45°59′36.44″N	84°21′3.69″W	3	CBMI
Chain Lake	Michigan	45°52′40.09″N	84°45′17.92″W	2	CLMI
Chalk Hill, Menominee River	Michigan	45°29′31.42″N	87°47′54.36″W	1	CHMI
Christmas Lake	Minnesota	44°53′53″N	93°32′29″W	3	CLMN
Clark Fork Driftyards	Idaho	48°10′36.65″N	116°14′7.29″W	1	CFID
Clear Lake	Ontario Canada	46°14′12.67″N	81°45′34.55″W	1	CLOC
Crystal Lake	Idaho	48°10′35.85″N	116°52′45.03″W	2	CLID
Fairfield Pond	Vermont	44°51′14.28″N	72°59′25.89″W	1	FPVT
Indian Lake	Connecticut	41°55′1.56″N	73°29′45.94″W	1	INCT
Kingsford Res., Menominee River	Michigan	45°49′18.22″N	88°08′07.79″W	2	KRMI
Lake Canadis	Ohio	41°8′53.71″N	81°26′50.27″W	2	LCOH
Lake Carroll	Illinois	42°10′49.62″N	89°52′53.69″W	1	LCIL
Lake Eligo	Vermont	44°35′58.51″N	72°21′22.73″W	3	LEVT
Lake Hodgson	Ohio	41°7′56.06″N	81°17′18.09″W	1	LHOH
Lake Ovid	Michigan	42°56′18.28″N	84°24′38.90″W	3	LOMI
Lake Scugog	Ontario Canada	44°33′44.43″N	78°8′39.25″W	2	LSOC
Little Bearskin	Wisconsin	45°42′37.57″N	89°42′4.74″W	3	LBWI
Luna Lake	Ohio	40°55′10.68″N	81°37′6.88″W	1	LLOH
McDill Pond	Wisconsin	44°30′6.66″N	89°32′56.62″W	4	MPWI
McFarlane Lake	Ontario Canada	46°24′57.91″N	80°57′47.34″W	1	MLOC
Michigamme Falls, Menominee River	Michigan	45°57′59.47″N	88°12′29.37″W	2	MFMI
Minocqua Lake	Wisconsin	45°52′26.17″N	89°41′40.97″W	2	MLWI
North Lake	Wisconsin	43°9′12.07″N	88°22′44.12″W	2	NLWI
Osoyoos Lake	Washington	48°58′36.29″N	119°26′19.201″W	1	OLWA
Otter Lake	Minnesota	44°53′18.28″N	94°24′32.89″W	4	OLMN
Peavy Falls, Menominee River	Michigan	46°00′12.76″N	88°12′37.54″W	1	PFMI
Pigeon Lake	Ontario Canada	44°33′25.94″N	78°30′0.63″W	1	PLOC
Richard Lake	Ontario Canada	46°26′14.47″N	80°54′57.86″W	2	RLOC
Spring Lake	Wisconsin	44°0′39.92″N	89°9′37.48″W	3	SLWI
Tripp Lake	Wisconsin	42°49′34.51″N	88°43′8.96″W	2	TLWI
White Rapids, Menominee River	Michigan	45°32′12.35″N	87°48′16.41″W	1	WRMI
Williams Lake	Wisconsin	43°45′44.09″N	89°22′31.88″W	3	WIWI

individuals. Specimens were placed live into ATL ® Qiagen (animal tissue lysis) buffer, were ground directly in a 1.5 mL centrifuge tube with a modified pipette tip, and were then stored at room temperature until DNA extraction. Some weevil samples collected remotely were preserved in water at –20 C until they could be processed in the lab. DNA was extracted with a DNeasy® Blood and Tissue kit (Qiagen®) following the protocol for purification of total DNA from insects.

Amplification of the partial mtDNA *cytochrome c oxidase subunit 1* (COI) sequence (~1085 bp) was conducted using the standard primers, LCO 5′GGTCAACAAATCATAAAGATAT-

TGG3′ (Hebert *et al.*, 2003) and C1N2776 5′GGATAATCAGAATATCGTCGAGG3′ (Simon *et al.*, 2006). Primers were used in final concentration of 1.25 μM. PCR amplification was conducted with 1μL DNA extract in a 25 μL reaction mixture using Promega HotStart *Taq* (1 μL of DNA; 10.5 μL H_20; 12.5 μL HotStart *Taq* mastermix; 0.5 μL forward primer; 0.5 μL reverse primer). Amplification conditions included an initial denaturation period of 2 min at 94 C , followed by 10 −1 C stepdown cycles which began at 55 C and ended at 45 C, then followed by 30 cycles of 94 C denaturation (30 s), 45 C annealing (30 s), and 72 C extension (60 s) with a final extension of 10 min. Purification and sequencing of the samples were completed by Annis Water Resources Institute (Muskegon, Michigan) with the same primers used in PCR amplification in an ABI 3130× sequencer. Forward and reverse sequences were assembled, manually reviewed, and edited with the software package Sequencher® (Gene Codes Corporation). Sequences were imported into MEGA5 (Tamura *et al.*, 2011) and aligned with the built-in automated aligner CLUSTALW. Sequences were trimmed to the shared 986 base pairs for the purposes of determining haplotypes and to ensure that the sequence was properly coding for amino acids.

Sequences were then uploaded to DnaSP v.5.10.01, a program which analyzes polymorphism data between sequences and outputs the haplotype file (Librado and Rozas, 2009). Based on the haplotype designations, a Maximum Likelihood Tree with 500 bootstraps was constructed in MEGA 5.2 (Tamura *et al.*, 2011). The best fit substitution model was determined to be Tamura 3-parameter + discrete gamma distribution + invariable sites in MEGA 5.2 (Tamura *et al.*, 2011). This model had the lowest Bayesian Information Criterion (BIC) score and therefore is considered to best describe the substitution model (Nei and Kumar, 2000). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was calculated in MEGA 5.2 (Tamura *et al.*, 2000) and was used to model evolutionary rate differences among sites [five categories (+*G*, parameter = 0.1597)]. The analysis involved 43 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. There were a total of 986 positions in the final dataset. Outgroups for the tree were chosen from the subfamily Ceutorhynchinae, to which *E. lecontei* belongs. *Parenthis vestitus* was determined to be the sister species of *E. lecontei* in a previous study (Roketenetz, 2015 unpub. or from GenBank. GenBank accession numbers for the outgroups are *P. vestitus* (KX789168), *Ceutorhynchus neglectus* (DQ058697.1), *C. erysimi* (DQ058698.1), and *C. gallorhenanus* (DQ058700.1). Each haplotype sequence was input into the online identification system for the Barcode of Life Database (BOLD) to verify percent similarity with known specimens in the database from Churchill, Manitoba, Canada and Fairbanks, Alaska (http://www.boldsystems.org/index.php/IDS_OpenIdEngine; Ratnasingham and Herbert, 2007). The haplotype file was also analyzed with Network v.4.6.1.2 which allowed for the creation of a haplotype network through the median joining calculation (Bandelt *et al.*, 1999; fluxus-engineering.com).

Location coordinates (x,y values; latitude, longitude) for the samples were input into ArcGIS v.10 and a distance matrix was created. In all cases the centroid of the waterbody where the sample was collected was used. A Mantel test was conducted in the program Alleles in Space (AIS; Miller, 2005). AIS also was used to determine the genetic barrier(s) across the landscape using Monmonier's Maximum Difference Algorithm.

A spatial analysis of molecular variance (SAMOVA) was conducted in SAMOVA 1.0 (Dupanloup *et al.*, 2002). This program uses a simulated annealing process repeated 100 times to determine which groups of populations are maximally genetically differentiated

TABLE 2.—Polymorphism summary statistics for total data set based on 984 bp of *Euhrychiopsis lecontei* COI mtDNA. Data were calculated in DnaSP v.5 (Librado and Rozas, 2009); and MEGA 5.2 (Tamura *et al.*, 2011)

No. of samples	No. of haplotypes	Average no. of nucleotide differences (k); SE	Haplotype diversity (h); SD	Nucleotide diversity (π); SD	Tajima's D; P-value
67	39	8.834; 1.695	0.958; 0.014	0.00898; 0.00075	-0.40230; $P > 0.10$

from each other, without previous assumptions regarding which individuals are assigned to each group (Eble *et al.*, 2011). The program was run for K = 2–10 groups to determine the most robust groupings of populations. The smallest K that gave the highest percent of variation explained by the proposed sample groupings (F_{ct}), that was not significantly different from a higher percentage of variation described by a higher K, was utilized (Eble *et al.*, 2011). A likelihood ratio test was utilized to determine the grouping value (K) that explained the greatest variation in the data. Following the assignation of population groups, Tajima D's statistic and its significance was calculated for each population grouping in DnaSP v.5.10.01 (Librado and Rozas, 2009) to test for signs of recent population expansion. Mismatch distributions were calculated for the whole data set and for each population grouping independently in DnaSP v.5.10.01 9 (Librado and Rozas, 2009) to obtain tau (τ). These results, along with mutation rates of 2.3% pairwise sequence divergence per million years (Brower, 1994) as the lower limit, 3.54% pairwise sequence divergence per million years (Papadopoulou *et al.*, 2010) as the upper limit, and a generation time of 0.25 y (Newman *et al.*, 2001 reports 3–6 generations per year), were used in a program that estimates time since divergence from substitution rates (http://www.uni-graz.at/zoowww/mismatchcalc/).

RESULTS

For the 67 individuals of *E. lecontei* sampled from 35 populations, 39 haplotypes were detected with an average number of nucleotide differences of 8.8 (Table 2). The analysis conducted by DnaSP v.5 (Librado and Rozas, 2009) utilized 984 of the 986 base pair sites (missing data were excluded). Haplotype diversity was high, whereas nucleotide diversity was low (Table 2). Tajima's D statistic was negative but not significant (Table 2).

Of the 984 base pair sites, 48 were polymorphic. Sixteen of these polymorphisms were singleton mutations with two variants and 32 were parsimoniously informative with two variants. No transversions were detected in the data set. The translated protein code (MEGA5; Tamura *et al.*, 2011) revealed 328 amino acids in the final data set. A single nonsynonymous substitution was detected in the translated protein code. A base pair change at position 331 in the sequence data (C → T) resulted in three haplotypes (H33, H34, and H38) having a serine (TCA) instead of a proline (CCA) at amino acid position 111.

The maximum likelihood tree indicates that all haplotypes of *E. lecontei* form a monophyletic group with 98% bootstrap support (Fig. 1). Within *E. lecontei*, most branch lengths (which measure the number of substitutions per site) were <0.0004. Based on a branch length of 0.008 and a 92% bootstrap support, the tree indicates there are two major groupings of haplotypes: Group A contains 27 haplotypes made up of 49 individuals from 26 populations (Fig. 1; Table 3); Group B contains 12 haplotypes made up of 18 individuals from nine populations (Fig. 1; Table 3). H37 is slightly separated from the other individuals in Group A by a branch length of 0.006 and 72% bootstrap support (Fig. 1).

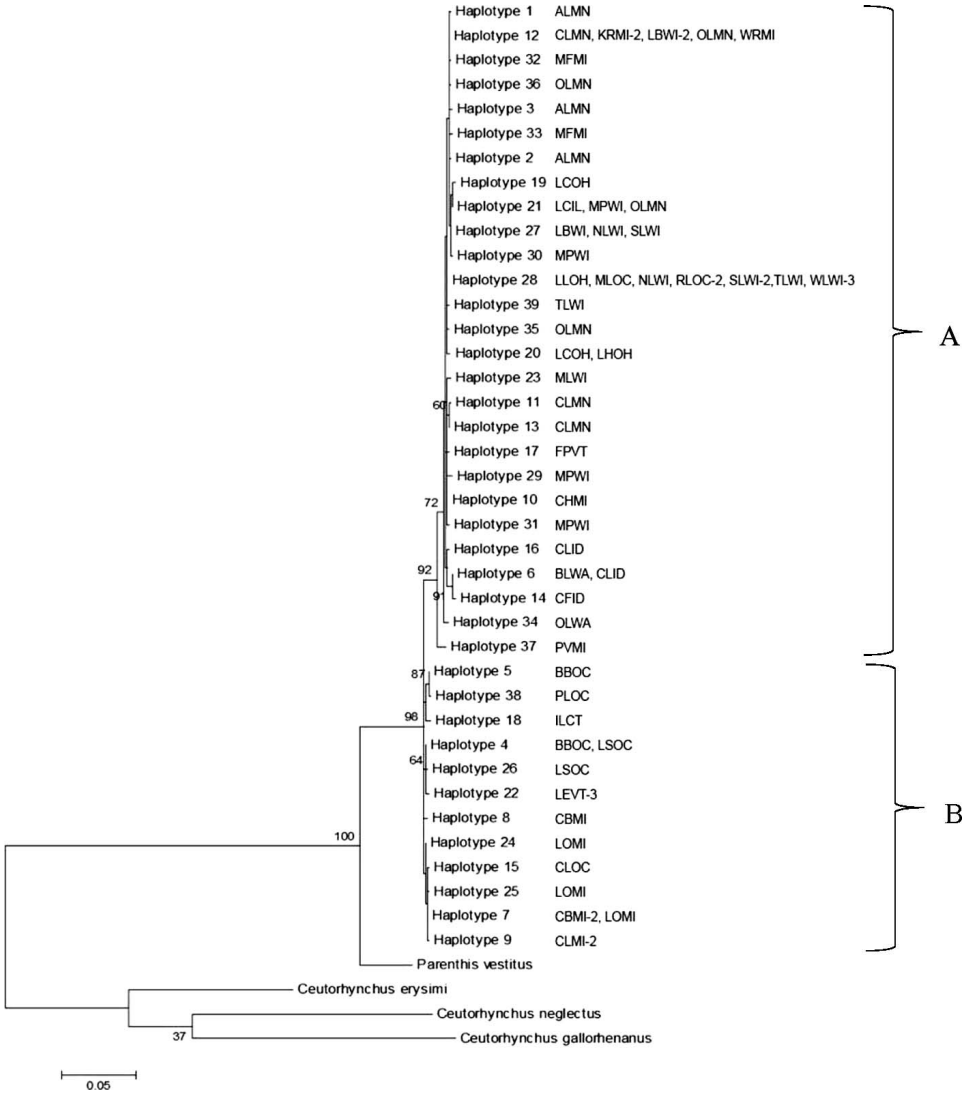

FIG. 1.—Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history of a portion of COI mtDNA from 67 individuals of Euhrychiopsis lecontei was inferred using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (–3338.7135) is shown. The percentage of trees (>50%) in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.,* 2011). Designation of Clades A and B are marked with brackets and the site codes are included

TABLE 3.—List of waterbodies, associated haplotypes, sample size of each haplotype/waterbody (N) and group assignation within each population

Waterbody site code	Haplotypes (number of individuals)	Group assignation*	GenBank accession numbers
ALMN	H1(1), H2(1), H3(1)	A	KX789101–KX789103
BBOC	H4(1), H5(1)	B	KX789104; KX789105
BLWA	H6(1)	A	KX789106
CBMI	H7(2), H8(1)	B	KX789107–KX789109
CLMI	H9(2)	B	KX789110; KX789111
CHMI	H10(1)	A	KX789112
CLMN	H11(1), H12(1), H13(1)	A	KX789113–KX789115
CFID	H14(1)	A	KX789116
CLOC	H15(1)	B	KX789117
CLID	H6(1), H16(1)	A	KX789118; KX789119
FPVT	H17(1)	A	KX789120
INCT	H18(1)	B	KX789121
KRMI	H12(2)	A	KX789122; KX789123
LCOH	H19(1), H20(1)	A	KX789124; KX789125
LCIL	H21(1)	A	KX789126
LEVT	H22(3)	B	KX789127–KX789129
LHOH	H20(1)	A	KX789130
LOMI	H7(1), H24(1), H25(1)	B	KX789132–KX789134
LSOC	H4(1), H26(1)	B	KX789135; KX789136
LBWI	H12(2), H27(1)	A	KX789137–KX789139
LLOH	H28(1)	A	KX789140
MPWI	H21(1), H29(1), H30(1), H31(1)	A	KX789141–KX789144
MLOC	H28(1)	A	KX789145
MFMI	H32(1), H33(1)	A	KX789146; KX789147
MLWI	H23(1)	A	KX789131
NLWI	H27(1); H28(1)	A	KX789148, KX789149
OLWA	H34(1)	A	KX789150
OLMN	H12(1), H21(1), H35(1); H36(1)	A	KX789151–KX789154
PFMI	H37(1)	A	KX789155
PLOC	H38(1)	B	KX789157
RLOC	H28(2)	A	KX789156; KX789158
SLWI	H27(1), H28(2)	A	KX789159–KX789161
TLWI	H28(1), H39(1)	A	KX789162; KX789163
WRMI	H12(1)	A	KX789164
WIWI	H28(3)	A	KX789165–KX789167

* Based on ML Tree, Haplotype Network and SAMOVA results

All haplotype sequences were uploaded to BOLD, which reported percent similarity to known specimens of *E. lecontei* ranging from 97.45%–99.81%. BOLD only positively confirms species identity for individuals with >99% similarity. Given this criterion, only some haplotypes from Group A and none from Group B were confirmed as *E. lecontei*.

The haplotype network shows two main groupings, which exactly match the designations of Groups A and B from the ML tree and are separated by 10 mutational steps (Fig. 2). One haplotype (H37) is separated from Group A by nine mutational steps (Fig. 2). Therefore, this haplotype appears to constitute a third type that is distinct from the other two groups.

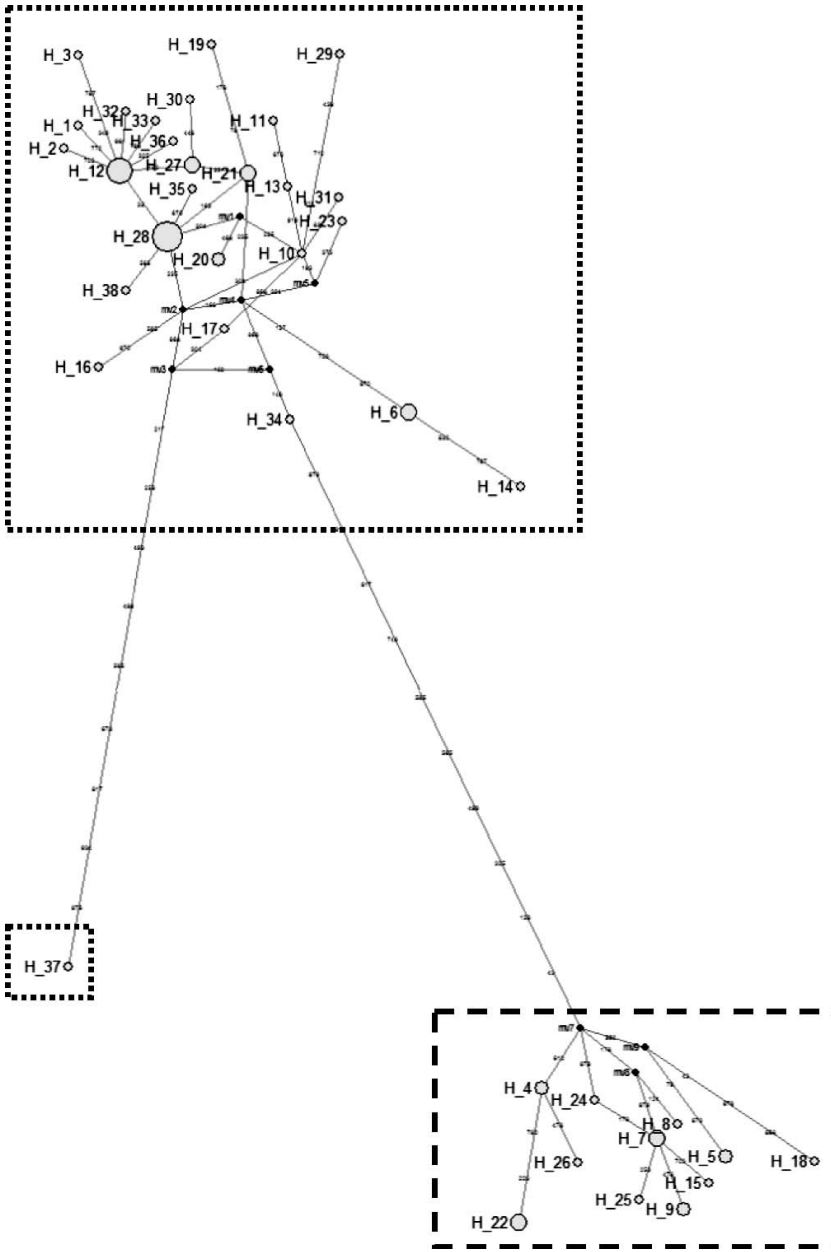

FIG. 2.—Haplotype Network of 39 haplotypes inferred from 984 base pairs of mtDNA COI gene for 67 individuals of *Euhrychiopsis lecontei*. Sample sizes ranged from one to four individuals per population. Small black nodes indicate missing intermediate haplotypes. Size of the gray nodes correlates to the number of individuals that share that haplotype. Clade A is designated by boxes with dotted lines and Clade B is designated by a box with dashed line

TABLE 4.—SAMOVA results for K = 2 groups

Source of variation	df	% of variation	Fixation indices	P-value
Between groups	1	77.6	$F_{ct} = 0.77629$	$P < 0.0001$
Among populations within groups	33	10.6	$F_{sc} = 0.47223$	$P < 0.0001$
Within populations	32	11.8	$F_{st} = 0.88193$	$P < 0.0001$

With K = 2, the SAMOVA results independently support the separation of the Groups A and B based on population locations (Tables 3, 4). Although higher Fct's were obtained with increasing K, the difference between K = 2 and K = 3 was not significant based on a Likelihood Ratio Test ($P > 0.75$). Therefore, the majority of the variation is between Groups A and B (Table 4).

Of the 984 sites used in the analysis, the number of variable sites within Group A was 37; 23 of these polymorphisms were singleton mutations with two variants and 14 were parsimoniously informative with two variants. Within Group B, 16 sites were variable; seven of these polymorphisms were singleton mutations with two variants and nine were parsimoniously informative with two variants. Between populations, there were two fixed differences: 30 mutations were polymorphic in Group A but monomorphic in Group B, nine mutations were polymorphic in Group B but monomorphic in Group A, and there were seven shared mutations. The average number of nucleotide differences between populations was 16.7. Haplotype diversity within each clade was high, while nucleotide diversity was low (Table 5). The average number of nucleotide differences, the haplotype diversity and the nucleotide diversity within each Group were similar to each other (Table 5). Both Groups A and B had negative Tajima D values, but only the value for Group A was statistically significant (Table 5).

The Mantel test conducted in AIS indicates there was a positive, significant correlation ($r = 0.223$, $P = 0.002$) between geographic and genetic distances based on 1000 replicates. Therefore, individuals that are geographically closer to each other are more likely to be more closely related genetically. Additionally, through Monmonier's Maximum Difference Algorithm, AIS predicted the geographic location of a barrier to gene flow. The predicted barrier matches the geographic separation already determined by the partitioning of Groups A and B through the SAMOVA analysis (Fig. 3)

Mismatch distributions calculated for Group A gave a $\tau = 1.578$ and for Group B, $\tau = 3.220$. Using mutation rates of 2.3% pairwise sequence divergence per million years (Brower, 1994) as the lower limit and 3.54% pairwise sequence divergence per million years (Papadopoulou *et al.*, 2010) as the upper limit, and a generation time of 0.25 y (Newman *et al.*, 2001 reports 3–6 generations per year), the online mismatch calculator (http://www.uni-graz.at/zoowww/mismatchcalc/mmc1.php) predicts that Group A's time since expansion is approximately 70,000 y ago and Group B's time since expansion is approximately 142,000 y ago.

DISCUSSION

Our broad scale genetic sampling of the aquatic weevil *E. lecontei* revealed substantial genetic structuring and no cryptic species. The phylogeographic patterns revealed in these data suggest the importance of glacial refugia in generating current patterns of genetic relatedness. We explore these patterns further below.

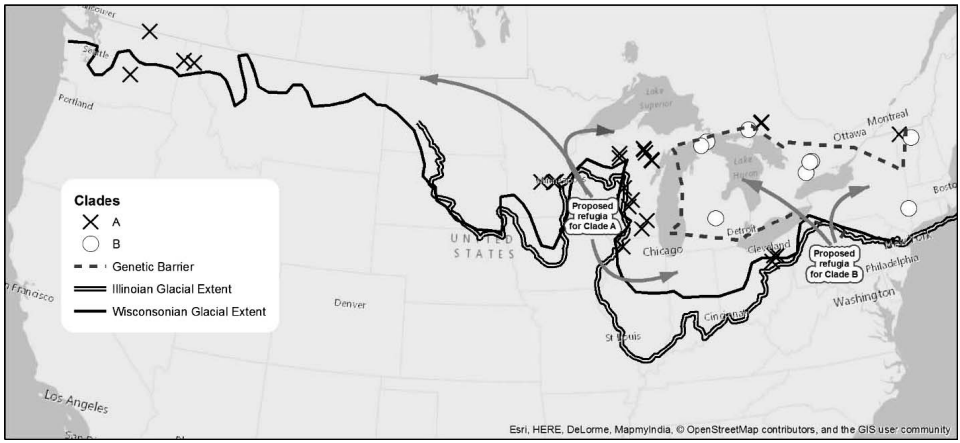

FIG. 3.—Proposed glacial refugia for Clades A and B. Arrows depict the possible colonization routes of *E. lecontei* into the distinct geographic areas delineated by the genetic barrier determined by our AIS analysis

Euhrychiopsis lecontei is represented by a monophyletic group, and therefore forms a single species as is shown in Figure 1 (consisting of haplotype groups A and B combined). As a group, haplotype diversity (H_d, Nei, 1987) was high indicating that most individuals within the data set have their own unique haplotype (Table 2; Adams and Villablanca, 2007). Nucleotide diversity (π) was low (Table 2) and tends to change more slowly than haplotype diversity (Adams and Villablanca, 2007). However, the combination of high haplotype diversity and low nucleotide diversity suggests that the population as a whole has undergone a relatively recent range expansion from a small remnant population (Avise, 2000; De Jong *et al.*, 2011).

No evidence of a cryptic morphological species is apparent. Based on the placement of the haplotypes that were >99.0% similar to know BOLD specimens within the maximum likelihood tree (Fig. 1), all haplotypes included in Group A can be confirmed as *E. lecontei*. Several haplotypes within Group B (H4, H7, H8, H9, H15, H24, H25) were >98% similar to the three known specimens of *E. lecontei* in BOLD. Although they cannot be definitively identified as *E. lecontei* based on this methodology, it is likely that increased inclusion of known specimens of this species from throughout their range into BOLD would allow for positive confirmation of the members of Group B as well. It should be noted H37 (Peavy Falls, Menonminee River, MI), the "outlier" in Figures 2 and 3, is one of the individuals with >99% similarity to the known *E. lecontei* specimens in BOLD. This individual is closely related to individuals from Manitoba, Canada, suggesting there may be more geographic structuring within *E. lecontei* than this study revealed, which could be confirmed with increased sampling efforts throughout its range (especially northern populations).

Genetic differentiation and geographic distance of *E. lecontei* populations exhibit a positive and significant correlation, supporting the notion of geographic structuring, and consistent with its limited dispersal capabilities. Newman *et al.* (2001) report weevils only disperse by flight in spring and fall when they are moving between the lake and their on-shore overwintering habitats. Furthermore, Newman *et al.* (2001) found overwintering *E. lecontei* individuals were generally only found 1–2 m from shore unless there are many lakes in close proximity, it is relatively unlikely *E. lecontei* is dispersing between lakes during fall/

TABLE 5.—Polymorphism summary statistics for Clades A and B based on 984 bp of *Euhrychiopsis lecontei* COI mtDNA. Data were calculated in DnaSP v.5 (Librado and Rozas, 2009); and MEGA 5.2 (Tamura *et al.*, 2011)

Clade	No. of samples	No. of haplotypes	Average no. of nucleotide differences (k); SE	Haplotype diversity (h); SD	Nucleotide diversity (π); SD	Tajima's D; P-value
A	49	27	3.561; 0.823	0.930; 0.024	0.00362; 0.00051	-1.94; $P < 0.05$
B	18	12	3.889; 1.118	0.948; 0.001	0.00395: 0.00075	-0.63; $P > 0.10$

spring flights. Rather, overwintering *E. lecontei* will often return to the same lake where they were born and perhaps even to the same Eurasian watermilfoil bed. In mid-summer flight muscles are extremely reduced making dispersal during the breeding season extremely limited (Newman *et al.*, 2001).

The positive relation between genetic differentiation and geographic distance is also congruent with the ecology of the weevil's hostplant (Eurasian watermilfoil), which tends to spread vegetatively through viable fragments after autofragmentation or mechanical fragmentation (Madsen and Smith, 1997). Fragments of Eurasian watermilfoil are often able to establish new populations once they settle onto the substrate after being dispersed by water flow or boat traffic, especially between closely situated waterbodies (Madsen and Smith, 1997). Because *E. lecontei's* life cycle is intimately tied to this plant, there is a great chance that viable fragments of Eurasian watermilfoil that move between waterbodies may also be transporting eggs, larvae, pupae, and/or adults of *E. lecontei*. Therefore, even with *E. lecontei's* limited dispersal capabilities through swimming or flight, they are likely able to achieve intermediate dispersal between hydrologically-linked waterbodies by "hitching a ride" on Eurasian watermilfoil fragments. However, it should be noted even populations separated by a great geographic distance (*e.g.*, those in Washington/Idaho vs. those in Minnesota/Wisconsin; *see* Tables 1, 3; Fig. 3) fall within the same haplotype group, suggesting there are other evolutionary forces, such as past glaciation events, that also play a role in the current geographic structuring of this weevil.

When examining the demographic history of each major *E. lecontei* haplotype group independently (Table 5), similar, but not identical, demographic histories can be inferred. Both groups have high H_d and low π, which indicates each clade has independently undergone a relatively recent range expansion from a small remnant population (Avise, 2000; De Jong *et al.*, 2011). When comparing the values from each group, H_d is significantly different between Group A and Group B, but π is only marginally significantly different (Group A does not overlap the mean of Group B, but Group B does overlap the mean for Group A) based on 95% confidence intervals. Furthermore, although both Groups A and B have negative Tajima's D values, they are only significantly negative for Group A. Negative Tajima D's can indicate the population has recently undergone a demographic expansion (Tajima, 1989). Several recent studies note star-like patterns in haplotype networks (*see* H12 and associated star pattern in Group A; Fig. 2) can also be indicative of a recent population expansion (Allcock and Strugnell, 2012; Ludt *et al.*, 2012; Ley and Hardy, 2014).

Together, these results suggest Group A has undergone a more recent range expansion than Group B. This is supported by predictions from the online mismatch calculator (http://www.uni-graz.at/zoowww/mismatchcalc/mmc1.php), which indicated the time since expansion for Group A was approximately 70,000 y ago and the time since

expansion for Group B was over two times longer (~142,000 y ago). It should be noted mutation rates can vary even within a group of organisms (Schenekar and Weiss, 2011); therefore, these expansion times should be viewed as general estimates. These dates fall within the Wisconsin glaciation (Group A) and Illinoian glaciation (Group B) of the Laurentide icesheet (Richmond and Fullerton, 1986) and support the view glacial dynamics likely play a role in the evolution of these geographically distant populations. The extents of the glacial maxima for these two glaciation events are shown in Figure 3.

The combined information from our results suggests the major *E. lecontei* haplotype groups have been isolated, potentially through several glacial and interglacial periods, and survived in isolated glacial refugia, as indicated in similar phylogenetic studies in another weevil species (acorn weevils, *see* Aoki *et al.*, 2008 and 2009). The geographic locations of Groups A and B also support this conclusion, as the proposed glacial refugia indicated in Figure 3 align well with two of the 10 proposed North American glacial refugia for terrestrial plants and animals (Beatty and Provan, 2010). Similar patterns have been shown for these two refugia in numerous species including smallmouth bass (Borden and Krebs, 2009), black spruce (Jamarillo-Correa *et al.,* 2004), jack pine (Godbout *et al.,* 2005), wood frog (Lee-Yaw *et al.,* 2008), and mountain avens (Tremblay and Shoen, 1999). The present study highlights the importance such glacial refugia can also have for maintaining geographic structure in aquatic organisms.

CONCLUSIONS

Multiple lines of evidence support the hypothesis kall individuals sampled from a broad geographic region throughout *E. lecontei's* range are of one species. No cryptic species were discovered through this study, although distinct geographic and genetic structuring was revealed, likely due to current geographic isolation of the sampled waterbodies, as well as the locations of historical glacial refugia. Further work should include more geographically widespread sampling throughout the U.S. and Canada and specifically targeted in areas where *E. lecontei* has not yet been recorded *(i.e.*, Montana, Wyoming, North Dakota, and South Dakota), not only for the increase in species-specific knowledge about this important weevil taxon, but also to better describe any geographic structuring. Additionally, because baseline genetic data has now been recorded for natural populations of *E. lecontei*, studies should be conducted on augmented biocontrol populations to verify rearing and deploying the insect has not degraded genetic variability and potentially its effectiveness, as a biocontrol agent (Mackauer, 1976). This project was important for confirming the species being utilized as *E. lecontei* in different biocontrol rearing operations is correctly identified as the intended biocontrol agent for *M. spicatum* (Eurasian watermilfoil). Incorrectly identified specimens used in such biological control programs would likely be unsuccessful, or perhaps unintentionally damaging to nontarget species, because such biocontrol programs rely on host specificity of the insect.

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SUBMITTED: 10 APRIL 2016 ACCEPTED 12 MAY 2017