Canada Warbler (Cardellina canadensis): novel molecular markers and a preliminary analysis of genetic diversity and structure

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ABSTRACT. The effects of predicted declines and potential loss of individual populations on species-level genetic diversity is unclear. A number of taxa, including the Canada Warbler (Cardellina canadensis), share wide-ranging geographic distributions in North American boreal forests with trailing-edge populations extending into the southern Appalachian Mountains. Trailing-edge populations in the southern portion of a species’ ranges often harbor high levels of genetic diversity and unique genetic variants, and may be at risk of extinction from climate change. Climate change and other anthropogenic factors are causing declines in the Canada Warbler’s southern trailing-edge populations, and with no genetic studies to date, the effect on species-level genetic diversity is uncertain. Species-specific microsatellite markers for the Canada Warbler were developed and validated using samples from three populations, including a southern trailing-edge population, to investigate their utility for intraspecific population studies. Eight of the microsatellite markers were informative for assessing genetic diversity and preliminary analysis suggests that they have potential for characterizing intraspecific neutral genetic diversity and structure among Canada Warbler populations.

La Paruline du Canada (Cardellina canadensis) : nouveaux marqueurs moléculaires et analyse préliminaire de la diversité et de la structure génétique

RÉSUMÉ. Les effets des baisses prédites et des pertes potentielles de certaines populations sur la diversité génétique à l'échelle de l'espèce sont incertains. Un grand nombre de taxons, dont la Paruline du Canada (Cardellina canadensis), partagent des répartitions géographiques très vastes dans les forêts boréales nord-américaines avec certaines populations s'étendant à la marge de l'aire de répartition jusque dans le sud des Appalaches. Ces populations situées à la limite sud de l'aire d'une espèce présentent souvent un degré élevé de diversité génétique et des variations génétiques uniques, et pourraient être à risque d'extinction à cause des changements climatiques. Les changements climatiques et d'autres facteurs d'origine humaine sont responsables de baisses dans les populations situées à la limite sud chez la Paruline du Canada, et en l'absence d'études génétiques jusqu'à présent, l'effet de la diversité génétique à l'échelle de l'espèce est incertain. Des marqueurs microsatellites spécifiques à la Paruline du Canada ont été élaborés et validés au moyen d'échantillons provenant de trois populations, y compris une population située à la limite sud, afin d'examiner leur utilité pour des études populationnelles intraspécifiques. Huit des marqueurs microsatellites se sont avérés instructifs pour évaluer la diversité génétique, et une analyse préliminaire indique qu'ils ont du potentiel pour caractériser la diversité et la structure génétique neutre intraspécifique parmi les populations de Paruline du Canada.

Key Words: Canada Warbler; Cardellina canadensis; climate change; genetic diversity; genetic structure; southern trailing-edge population

INTRODUCTION

In North America, the ranges of many species are shifting toward higher latitudes and upward in elevation in response to recent climate change (Parmesan 2006, Zuckerberg et al. 2009, Ralston and Kirchman 2013, Mason et al. 2015). Populations unable to respond to rapid environmental changes through range shifts or adaptation will be at risk of extinction, which may cause an overall loss of genetic diversity species-wide (Hughes et al. 1997, Davis and Shaw 2001, Dawson et al. 2011, Frankham 2005, McInerny et al. 2009, Pauls et al. 2013). Furthermore, loss of genetic diversity will likely be underestimated if cryptic diversity and intraspecific genetic variation are not considered (Pauls et al. 2013). Populations at the leading-edge of a range expansion may have relatively low levels of genetic diversity due to founder effects (Cobben et al. 2011, Arenas et al. 2012). Differences in genetic diversity between central and peripheral populations may be slight (Eckert et al. 2008), and in some cases, regional genetic diversity of trailing-edge populations can be high, especially when these populations exist near glacial refugia that were relatively stable during Pleistocene climate oscillations (Tzedakis et al. 2002, Vucetich and Waite 2003, Hewitt 2004, Eckert et al. 2008). Furthermore, decreased gene flow among peripheral populations can lead to pronounced genetic structure (Bohonak 1999), and trailing-edge populations may harbor unique alleles not found in...
other regions of the range (Petit et al. 2003, Hewitt 2004, Provan and Maggs 2012). The possibility that some trailing-edge populations were diverging from populations at higher latitudes prior to recent environmental change (Hewitt 1996, Hampe and Petit 2005, Parisod and Joost 2010) has led to calls for their recognition as distinct ecological and evolutionary units (Crancell et al. 2000, Fraser and Bernatchez 2001).

The Canada Warbler (Cardellina canadensis) is a Neotropical-Nearctic migratory species of conservation concern that, according to the North American Breeding Bird Survey (BBS), has been declining at a rate of 2.3% per year since 1966 (Sauer et al. 2014). Although BBS data are not sufficient for reliably estimating historic trends of peripheral Canada Warbler populations in the northern boreal or southern Appalachian Mountain regions, species distribution models predict widespread population declines near the southern edge of the breeding range (Matthews et al. 2004, Sauer et al. 2014). Population declines and model projections have prompted organizations such as the North American Bird Conservation Initiative, Partners in Flight, and the Northeast Endangered Species and Wildlife Diversity Technical Committee to designate Canada Warblers as high priority for research and conservation (Reitsma et al. 2010, Stralberg et al. 2015, 2017). In Canada, this species is listed as Threatened under the Species at Risk Act (S.C. 2002, c.29; Environment Canada 2016). The National Audubon Society has classified Canada Warbler as one of 188 North American bird species that is expected to lose more than 50% of its geographic range by 2080 because of climate change (Langham et al. 2015).

The breeding range of the Canada Warbler extends across the southern boreal region of Canada into the northeastern United States, including the Great Lakes region and Appalachian Mountains into northeastern Georgia (Reitsma et al. 2010). Southern Appalachian populations are patchily distributed and restricted to habitats above 1000 m elevation (COSEWIC 2008, Reitsma et al. 2010). Canada Warblers utilize various habitats throughout their range, but are most common in moist, mixed coniferous-deciduous forests (Reitsma et al. 2010, Haché et al. 2014, Ball et al. 2016). As ground nesters, they are dependent on complex forest structure, especially dense understory vegetation (Hallworth et al. 2008, Goodnow and Reitsma 2011, Becker et al. 2012). Adult Canada Warblers display high breeding site fidelity (Hallworth et al. 2008), which has been documented as a contributor to limited gene flow and strong population structure in other songbird species (Temple et al. 2006, Coulon et al. 2008, Walsh et al. 2012). The extent of natal dispersal is largely unknown, although it can be as short as 500 m in North Carolina and New Hampshire (RBC and LRR, unpublished data).

No population studies examining genetic diversity and structure have been conducted for the Canada Warbler and no molecular markers for the species have been reported. Development of neutral molecular markers for the species will facilitate studies examining genetic diversity and connectivity across the Canada Warbler’s range. It is unclear how the loss of peripheral populations might impact species-level genetic diversity, therefore, characterizing genetic diversity and distribution for the species is needed to inform conservation and management strategies. To address this need, we developed novel species-specific microsatellite markers for Canada Warbler and conducted a preliminary analysis to evaluate their utility for intraspecies population studies. We compared a leading- and a trailing-edge breeding population to a population located within the central portion of the species’ breeding range. We hypothesized that southern populations would have high levels of genetic diversity because they occur near glacial refugia in the southern Appalachian Mountains and that the three populations would be genetically distinct because of geographic distances between them.

**METHODS**

**Sample collection**

Rectrices were collected from Canada Warblers breeding in three study areas: the United States Department of Agriculture (USDA) Coweeta Hydrologic Laboratory in Otto North Carolina, U.S. (NC, n = 72), Canaan, New Hampshire, U.S. (NH, n = 57), and Fort Liard in the Northwest Territories, Canada (NWT, n = 40) during the 2014 and 2015 breeding seasons (Fig. 1). Pairwise geographic distances between Coweeta NC-Canaan NH, Coweeta NC-Fort Liard NWT, and Canaan NH-Fort Liard NWT are 1371 km, 3997 km, and 3856 km, respectively. Individuals were caught by mist net and were banded with United States Geological Survey (USGS) aluminum bands. At the NC site, approximately 100 μL of blood was collected from the brachial vein and stored on Flinders Technology Associates (FTA™) cards or in lysis buffer. Samples placed on FTA™ cards were allowed to dry and stored at -20 °C. Samples in lysis buffer were stored at room temperature. Two rectrices were obtained from each individual and placed in small envelopes, which were stored at room temperature or -20 °C. Samples collected in NH and Canada were subsequently transferred to 95% ethanol for preservation and transport.

**Fig. 1.** Distribution map of the breeding and migratory range for Canada Warblers (Cardellina canadensis). Sample collection sites are indicated on map with lettered circles: (A) Coweeta, North Carolina, USA; (B) Canaan, New Hampshire, USA; (C) Fort Liard, Northwest Territories, Canada. (Map from The Birds of North America https://birdsna.org; used with permission)
Table 1. Characterization of microsatellite markers developed for Canada Warblers (Cardellina canadensis) tested on samples from a single population in Coweta, North Carolina. N, number of individuals genotyped at each locus; k, number of alleles at each locus; $H_o$, observed heterozygosity; $H_e$, expected heterozygosity; and $P_{HW}$, probability that genotype proportions conform to Hardy-Weinberg Proportions. GenBank accession numbers are listed for each microsatellite locus sequence.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer Sequence</th>
<th>Motif</th>
<th>N</th>
<th>k</th>
<th>Size range (bp)</th>
<th>$H_o$</th>
<th>$H_e$</th>
<th>$P_{HW}$</th>
<th>GenBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAWA05</td>
<td>F: 5'-GGTCCCTTCATGATGTGCC</td>
<td>(ATATC)$^a$</td>
<td>71</td>
<td>9</td>
<td>232-272</td>
<td>0.732</td>
<td>0.768</td>
<td>0.6571</td>
<td>KY924652</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AGCCCTCTGGATCTTCTCCG</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAWA11</td>
<td>F: 5'-ATGAACTCTGGGCTGTGCTG</td>
<td>(AACAT)$^b$</td>
<td>70</td>
<td>10</td>
<td>152-202</td>
<td>0.714</td>
<td>0.867</td>
<td>&lt; 0.0001</td>
<td>KY924653</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTCTGTGACCTCTTTCC</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>CAWA13</td>
<td>F: 5'-CTCTAAGGCGACGAAAGC</td>
<td>(ATGCC)$^c$</td>
<td>72</td>
<td>14</td>
<td>389-454</td>
<td>0.917</td>
<td>0.898</td>
<td>0.7964</td>
<td>KY924654</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GCCTAGACGACGCTTGGG</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CAWA20</td>
<td>F: 5'-ACCTGTACCTTTTCCTCGC</td>
<td>(AGAT)$^d$</td>
<td>71</td>
<td>8</td>
<td>375-403</td>
<td>0.803</td>
<td>0.813</td>
<td>0.8833</td>
<td>KY924655</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AGGTGTTACATTGGCGAGT</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>CAWA25</td>
<td>F: 5'-ACCTGCTGTGACCTTG</td>
<td>(ATCC)$^e$</td>
<td>71</td>
<td>11</td>
<td>112-196</td>
<td>0.648</td>
<td>0.835</td>
<td>&lt; 0.0001</td>
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<tr>
<td></td>
<td>R: 5'-ACCTAAAGACCTCTGCGAC</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>†CAWA28 F: 5'-GGACACCATGCTTGCTC</td>
<td>(AAAG)$^f$</td>
<td>71</td>
<td>32</td>
<td>264-484</td>
<td>0.915</td>
<td>0.956</td>
<td>&lt; 0.0001</td>
<td>KY924657</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AAACGTCTGTGTTTGCGT</td>
<td></td>
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<tr>
<td></td>
<td>†CAWA29 F: 5'-GGACCTCTGGAGAATCCTG</td>
<td>(AAATAG)$^g$</td>
<td>70</td>
<td>23</td>
<td>435-546</td>
<td>0.871</td>
<td>0.935</td>
<td>0.2310</td>
<td>KY924658</td>
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<tr>
<td></td>
<td>R: 5'-CTTTCACAGCCGGTTGTAC</td>
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<tr>
<td></td>
<td>†CAWA30 F: 5'-GGTTTCACACACATCCCTC</td>
<td>(AAAGA)$^h$</td>
<td>69</td>
<td>29</td>
<td>285-450</td>
<td>0.942</td>
<td>0.945</td>
<td>0.9843</td>
<td>KY924659</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GGATGGACAGAGGGTATGGC</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>†CAWA33 F: 5'-TTCTTTGTGCCCTTGCG</td>
<td>(AAGAG)$^i$</td>
<td>72</td>
<td>12</td>
<td>214-274</td>
<td>0.875</td>
<td>0.855</td>
<td>0.8055</td>
<td>KY924660</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AAGATGGACAGGGCTCTCAG</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>†CAWA37 F: 5'-GCTCCCTACTGCGCATAG</td>
<td>(AGATG)$^j$</td>
<td>72</td>
<td>19</td>
<td>290-456</td>
<td>0.75</td>
<td>0.913</td>
<td>&lt; 0.0001</td>
<td>KY924661</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CCTCTAATGTCACCTTGCC</td>
<td></td>
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</tr>
</tbody>
</table>

$^a$CAG Tag (CAGTCGGGCTTGACAT)$
$^b$GTT Tag (GTTT)$
$^c$M13 Tag (GGAAACACATGCTCTTGCCCT)
$^d$Markers that were removed prior to analyses because of high null allele frequencies.

DNA extraction and analysis

Total genomic DNA was isolated from individual samples of either 10 µL of blood or single feathers using the DNEasy Blood and Tissue® kit (Qiagen). Feather samples were macerated prior to digestion. Individual DNA samples were genotyped using the 10 locus-specific primer pairs. Amplification reaction conditions, thermal cycling parameters, fragment analyses, and allele scoring were conducted as previously described in Tumas et al. (2017).

Primer development

Novel microsatellite markers were developed for population genetic studies of Canada Warbler. Genomic DNA was isolated from a blood sample, obtained from a Canada Warbler captured in NC, using a DNEasy Blood and Tissue® kit (Qiagen). Total genomic DNA was used for library preparation using the Kapa Biosystems® KAPA LTP Library Preparation Kit for Illumina® platforms. Paired-end sequences were analyzed using the Illumina® NextSeq sequencing system. Paired reads were assembled using Geneious 8.1.6 (Kearse et al. 2012) and sequences over 150 bp in length were extracted and queried for microsatellite loci using MSATCOMMANDER (Faircloth 2008). Of the 3300 microsatellites identified, 1100 included unique sequences surrounding the repeats with sufficient length for primer design. Forty-eight primers were selected using repeat length, motif, number of repeating units and primer pair penalty. Primers were tagged as noted (Table 1). A M13, CAG, or GTTT tag was added to the 5’ terminus of locus-specific primers and amplification reactions carried out as described in Tumas et al. (2017). The 48 primer pairs were screened for amplification consistency and polymorphism in 30 Canada Warblers captured at the NC study site. Loci were not tested for sex linkage and all loci that produced inconsistent amplification or null alleles were excluded. Ten primer pairs amplified consistently in these samples and were subsequently used to genotype samples from the three populations (Table 1).

Genetic diversity

Allelic data were scored using GeneMapper® 5.0 (Applied Biosystems) and formatted for analysis using GMCONVERT (Faircloth 2006). CERVUS 3.0 (Kalinowski et al. 2007) was used to calculate mean number of alleles per locus (k), observed heterozygosity ($H_o$), expected heterozygosity ($H_e$), and deviations from Hardy-Weinberg Proportions ($P_{HW}$). Linkage disequilibrium (LD) was calculated using GENEPOP (Raymond and Rouset 1995; Table 1). Arlequin (Excoffier et al. 2005) was used to calculate $H_o$, $H_e$, and k for each study area. Number of private alleles was calculated in R using the poppr package (Kamvar et al. 2014, R Core Team 2016). Allelic richness (A_R) with rarefaction to the minimum population sample size (n) was calculated in R using the divBasic function in the “diversity package” (Keenan et al. 2013, R Core Team 2016). Significant differences in observed heterozygosity and allelic richness were tested between pairs of the three populations using a two sample t-test across locus values for each metric in R.

Population differentiation

Estimates of pairwise FST values were calculated using Arlequin (Excoffier et al. 2005). STRUCTURE 2.2 (Pritchard et al. 2000) was used to estimate the number of genetically distinct clusters. Both the admixture and the no admixture model were implemented in STRUCTURE (250,000 burnin, 250,000
additional MCMC iterations) with correlated allele frequencies, using sampling locations as a prior (locprior) as well as without sampling locations as a prior, and otherwise default settings. We ran 20 iterations for each hypothesized number of genetic clusters (K) ranging from 1 to 6. The optimal K was evaluated using two methods, the Evanno method (Evanno et al. 2005) and the ln-likelihood method as described in Rosenberg et al. (2001) using the average probability per value of K. STRUCTURE HARVESTER (Earl and vonHoldt 2012) was used to evaluate the optimal K according to the method described in Evanno et al. (2005).

RESULTS

Primer development
Forty-eight primers pairs were tested for amplification using 30 Canada Warbler samples from the NC study site. Ten primer pairs consistently amplified the respective locus. Two markers (CAWA28 and CAWA37) of the 10 microsatellite markers validated in this study produced high null allele frequencies that could not be resolved with subsequent regenotyping. These two loci were excluded from subsequent analyses (Table 1).

Genetic diversity
The number of alleles per locus for the 169 genotyped individuals ranged from 8 to 32. Overall, populations had an average observed heterozygosity \(H_O\) of 0.80, and an average allelic diversity (k) of 17.5 (Table 2). Observed heterozygosity was similar for the three populations (NC = 0.80, NH = 0.80, NWT = 0.78). Allelic richness was highest in the NH population (13.6), intermediate in the NC population (12.8), and lowest in the NWT population (11.6). Observed heterozygosity and allelic richness were not significantly different between any pair of populations based on a two sample t-test (p > 0.05). The total number of private alleles was 27, with 16 alleles in the NH population, 10 identified in the NC population, and one in the NWT population.

Table 2. Number of individuals genotyped (N), observed heterozygosity \(H_O\), expected heterozygosity \(H_E\), mean number of alleles per locus (k), and private alleles.

<table>
<thead>
<tr>
<th>Population</th>
<th>N</th>
<th>(H_O)</th>
<th>(H_E)</th>
<th>k</th>
<th>(A_k)</th>
<th>Private Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>72</td>
<td>0.80</td>
<td>0.86</td>
<td>14.5</td>
<td>12.8</td>
<td>10</td>
</tr>
<tr>
<td>NH</td>
<td>57</td>
<td>0.80</td>
<td>0.87</td>
<td>15.1</td>
<td>13.6</td>
<td>16</td>
</tr>
<tr>
<td>NWT</td>
<td>40</td>
<td>0.78</td>
<td>0.87</td>
<td>12.3</td>
<td>11.6</td>
<td>1</td>
</tr>
<tr>
<td>Overall</td>
<td>169</td>
<td>0.80</td>
<td>0.87</td>
<td>17.5</td>
<td>NA</td>
<td>27</td>
</tr>
</tbody>
</table>

Population differentiation
Pairwise estimates of \(F_{ST}\) indicated that the NC population was significantly different from NH and NWT (\(F_{ST} = 0.008, p < 0.0001\) for both comparisons), but the NH and NWT populations were not significantly different (\(F_{ST} = -0.002, p = 0.883\)). The two methods used to identify the optimal K from the STRUCTURE analyses differed in their results. The Evanno method indicated K = 2, which was consistent with STRUCTURE bar plots generated from the locprior runs that indicated a NC cluster and a NH/NWT cluster (Fig. 2). However, structure was not apparent in the bar plot for K = 2 for the model runs that did not include sampling locations as a prior. The ln-likelihood method suggested all samples belonged to a single cluster, irrespective of whether sampling location was included as a prior.

Fig. 2. STRUCTURE output using the Evanno method (Evanno et al. 2005) and STRUCTURE HARVESTER (Earl and vonHoldt 2012) with (A) sampling locations as a prior and (B) without sampling locations as a prior. Sample sites are indicated on the X axis. NC: Coweeta, North Carolina, USA; NH: Canaan, New Hampshire, USA; NWT: Fort Liard, Northwest Territories, Canada.

DISCUSSION
An understanding of genetic diversity and structure is essential for the development of science-based conservation and management strategies for species threatened by habitat loss and climate change. Studies suggest that although neutral genetic diversity may not be correlated with adaptive genetic diversity, neutral genetic variation is an important consideration in conservation (Bonin et al. 2007, Moritz 1994) and molecular markers are effective tools for investigating gene flow, migration, and dispersal among populations (Holderegger et al. 2006).

Consistent with our hypotheses, we found evidence of possible population structure, with the southern trailing-edge population (North Carolina) being genetically distinct from the central (New Hampshire) and leading-edge (Northwest Territories) populations. In contrast to our predictions, the central and leading-edge populations did not differ from one another in spite of the large geographic distance between them. The Northwest Territories population did, however, have fewer unique alleles, consistent with a more recently established population.

Subtle genetic structure was present among the sampled Canada Warbler populations. Pairwise \(F_{ST}\) estimates indicated low but significant differentiation between NC and the two other populations (\(F_{ST} = 0.008, p < 0.0001\) for both comparisons). The Evanno method interpretation of STRUCTURE analyses using sampling locations as a prior (locprior) also supported the presence of two gene pools (K = 2), with individuals sampled in North Carolina assigned to one cluster, and individuals from NWT and NH assigned to a second cluster (Fig. 2). Without using
sampling location as a prior (nolocprior), the Evanno method interpretation of STRUCTURE analysis did not support K = 2. Analyses using the ln-likelihood method indicated the presence of a single gene pool.

This study suggests the possibility that the southern trailing-edge population of Canada Warblers may be genetically distinct from central and leading-edge populations and harbor unique genetic diversity. Although more comprehensive sample coverage and analyses are needed, these observations raise the possibility that extirpation of populations in the southern range margin could have negative genetic consequences for the species overall. Climate-based distribution modeling predicts range shifts for 15 boreal bird species under two carbon emissions scenarios (Ralston and Kirchman 2013) and other models predict poleward and upslope shifts with declines or extirpation of high elevation bird populations (Rodenhause et al. 2007, Virkkala et al. 2008). Trailing-edge populations of Canada Warblers already occur at the highest elevations in much of the southern Appalachians, so shifts in elevation are not possible. Species can persist in new conditions brought on by climate change through adaptation (Hoffmann and Sgrò 2011) or shifting their range (Chen et al. 2011). However, if the environment is changing more rapidly than the species can respond with adaption or range shifts, this may lead to extirpation (Davis and Shaw 2001, Dawson et al. 2011, Stralberg et al. 2015). This may also be the case for much of the biodiversity found in the southern Appalachian Mountains, which represents the low latitude range limit of numerous taxa (Stein et al. 2000).

Trailing-edge populations of Canada Warblers may be genetically distinct from populations at higher latitudes in the central and leading-edge of the species’ range, and harbor unique genetic diversity. However, more comprehensive sampling throughout the breeding range is needed to gain a more complete understanding of the spatial distribution of genetic diversity. Future studies should also attempt to understand the extent to which divergence of southern Appalachian populations is adaptive or the result of drift (Weeks et al. 2016).

Responses to this article can be read online at: http://www.ace-eco.org/issues/responses.php/1176

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LITERATURE CITED


