Molecular relationships of introduced *Aedes japonicus* (Diptera: Culicidae) populations in British Columbia, Canada using mitochondrial DNA

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ABSTRACT: *Aedes japonicus japonicus* (Theobald) is a relatively recent immigrant to the Pacific Northwest, having been collected in Washington State in 2001 and in British Columbia (BC) since 2014. We applied a molecular barcoding approach to determine the phylogenetic relationship of *Ae. j. japonicus* populations in BC with those from around the world. We sequenced a 617 base-pair segment of the cytochrome c oxidase 1 gene and a 330 base-pair region of the NADH dehydrogenase 4 gene to find genetic variation and characterize phylogenetic and haplotypic relationships based on nucleotide divergences. Our results revealed low genetic diversity in the BC samples, suggesting that these populations arose from the same introduction event. However, our approach lacked the granularity to identify the exact country of origin of the *Ae. j. japonicus* collected in BC. Future efforts should focus on detecting and preventing new *Ae. j. japonicus* introductions, recognizing that current molecular techniques are unable to pin-point the precise source of an introduction. ***Journal of Vector Ecology* 45 (2): 285-296. 2020.**

Keyword Index: Aedes japonicus, mitochondrial DNA, barcoding, polymorphism, cytochrome c oxidase 1, invasive species.

INTRODUCTION

The Asian rock pool mosquito, *Aedes* (Finlaya) *japonicus* (Theobald), is a day-biting mosquito commonly found in forested areas of East Asia (Tanaka et al. 1979). As larvae they are found in tree holes, rock pools, and artificial containers (Tanaka et al. 1979, Kaufman and Fonseca 2014). This species overwinters as eggs in colder areas and as larvae in warmer areas (Kampen and Werner 2014). Adult *Ae. japonicus* have distinct lyre-shaped bands of yellowish-brown scales on the scutum, basal bands of pale scales on hind tarsomeres 1-3, a variable hind tarsomere 4, and a dark-scaled hind tarsomere 5 (Tanaka et al. 1979). The spread of this container breeding species is believed to be a result of accidental transport in commercial shipments around the world. *Aedes japonicus* is native to Japan, Taiwan, Hong Kong, Northeast Russia, parts of China, and the Korean peninsula (Petrishcheva 1948, Tanaka et al. 1979). In 1998 this species was detected in North America in the states of New Jersey, New York, and Connecticut (Kampen and Werner 2014). Presently, *Ae. j. japonicus* has spread to much of the continental United States (Kampen and Werner 2014, Kaufman and Fonseca 2014, Riles et al. 2017, Bradt et al. 2018) and Canada (Thielman and Hunter 2006, Fielden et al. 2015). This species was collected on the island of Hawaii over a decade ago (Larish and Savage 2005) and more recently on the islands of Kauai and Oahu (Harwood et al. 2018).

Many invasive mosquitoes have caused or have the potential to cause major disease epidemics, such as the yellow fever outbreaks from Philadelphia to the tropics transmitted by *Aedes aegypti* in the past two centuries (Gubler 2004). *Aedes japonicus* is known to serve as a vector for Japanese encephalitis virus in Asia, as well as La Crosse Encephalitis virus and West Nile virus in North America (Kampen and Werner 2014, Harris et al. 2015, Kuwata et al. 2015). In laboratory settings *Ae. japonicus* has also shown vector competence for eastern equine encephalitis virus, St. Louis encephalitis virus, Rift Valley fever virus, chikungunya virus, dengue virus, and Getah virus (Kampen and Werner 2014). Understanding the origins of invasive *Ae. japonicus* populations is important to determine if this species is coming in from areas where these pathogens occur, and to predict potential impacts on human health associated with this mosquito.

As a species, *Ae. japonicus* comprises four morphologically identical but genetically distinct subspecies (*Ae. j. japonicus, Ae. j. shintienensis, Ae. j. yaeyamensis,* and *Ae. j. amamiensis)* (Cameron et al. 2010). The latter three subspecies have not been collected beyond East Asia while *Ae. j. japonicus* populations have expanded beyond Asia to Europe and North America (Kaufman and Fonseca 2014). Genetic evidence using mtDNA suggests that the establishment of *Ae. j. japonicus* in the eastern United States is the result of at least two independent introductions, and follow-up genetic studies also using mtDNA indicated that these established populations are now merging and interbreeding (Kaufman and Fonseca 2014). In Canada, adults were first identified in Ontario and Quebec in 2001 (Thielman and Hunter 2006, Savignac et al. 2002 in Kampen and Werner 2014). The species was found farther east, in New Brunswick, by 2008 and reached St. John's Newfoundland, the most easterly location in North America, by 2013 (Fielden et al. 2015). *Aedes j. japonicus* was found in King County Washington State in 2001, approximately 2000 km from the nearest recorded *Ae. japonicus* population (Roppo et al. 2004). The origin of this population and the time it established in Washington is unknown, but six years later *Ae. j. japonicus* was found in southern Washington and Oregon (Irish and Pierce 2008).

North America is not the only region in which *Ae. j. japonicus* has established outside of its native range. In the 1990s it was repeatedly intercepted in used tires arriving in

New Zealand (Laird et al. 1994). In Europe *Ae. j. japonicus* was first found in vehicle tires in Normandy and was promptly eradicated (Schaffner et al. 2003). Adults and larvae were detected in tires in Belgium in 2002, 2007, and 2008 (Versteirt et al. 2009). In 2008 this species was found in Switzerland and Germany (Schaffner et al. 2009), in 2011 it was found in Austria and Slovenia (Seidel et al. 2012), and since then it has been found in the Netherlands (Zielke et al. 2015), Hungary and Liechtenstein (Seidel, Nowotny et al. 2016), Italy (Seidel, Montarsi et al. 2016), and Croatia (Klobučar et al. 2018).

In 2014, *Ae. j. japonicus* was collected in Maple Ridge, an eastern suburb of Vancouver, BC, nearly 130 km north of the closest known *Ae. j. japonicus* population in Marysville, Washington (Jackson et al. 2016). This species has now been collected at other locations in British Columbia, one being within 20 km of the original Maple Ridge site (McCann 2015, unpublished), Burnaby, BC in 2016 (Peach 2018), and more recently in 2018, in Saanichton, BC (Peach 2018) marking the first documented detection of this species on Vancouver Island. While the presence of these mosquitoes in such heavily populated areas raises many concerns about future arbovirus transmission potential, these seemingly disjunct and newly established populations also present interesting opportunities to study the invasion dynamics of this highly invasive species.

Genetic diversity characterization allows us to monitor new arrivals of invasive species by identifying genetic variations between and within populations. Determining genetic relationships depends on the presence of high-quality genetic material, reliable and comprehensive reference datasets with comparable genetic sequences, as well as wide geographical survey range (Casiraghi et al. 2010, Schmidt et al. 2020). Over the last decade, mitochondrial DNA (mtDNA) sequences have been used extensively in population genetic studies. The most common mtDNA locus used in such studies is the highly conserved cytochrome oxidase 1 (COI) gene. The second most popular marker used in *Ae. japonicus* sequencing studies is the NADH dehydrogenase subunit 4 (ND4) gene, which has been used successfully in population level analysis (Cameron et al. 2010, Zielke et al. 2015). Both of these markers are maternally inherited and have been shown to segregate rapidly between generations leading to high rates of polymorphism (Gorrochotegui-Escalante et al. 2000, Zhong et al. 2013). Newer, higher resolution techniques such as genome wide single nucleotide polymorphism (SNP) and microsatellite profiling that examine thousands of genetic loci have allowed for an increased granularity to successfully delineate populations, especially in *Aedes* sp. (Gloria-Soria et al. 2014, Gloria-Soria et al. 2018, Schmidt et al. 2019, Schmidt et al. 2020) and should be considered the current best practice for species source identification.

The primary goal of this study was to compare the phylogenetic relationship of *Ae. j. japonicus* populations in British Columbia, Canada with those from around the world. Our samples represent the first sequences from Western Canada to be added to the growing *Ae. j. japonicus* dataset and thus contribute to future studies of mosquito ecology and evolution. Here, we used the sequence divergences of the CO1 and ND4 genes to find genetic variations and characterize haplotypic and phylogenetic relationships of *Ae. j. japonicus* from BC, Everett, and Washington with sequences from around the world. The goal of our study was to identify the origin of the BC populations.

MATERIALS AND METHODS

Mosquito sampling

In British Columbia, *Ae. j. japonicus* samples were collected in Maple Ridge and Saanichton as larvae, reared in the laboratory, and transferred, as adults, to 95% ethanol. Samples from Burnaby were collected as adults in Biogents Sentinel traps (Biogents AG, Regensburg, Germany) baited with dry ice and a Biogents scent lure. The single larva from Everett, Washington was collected at Granite Falls, Washington, and transferred to 95% ethanol. Adult specimens from Hamilton, Ontario were provided by CULEX Environmental (http://culex.ca/) in vials containing 75% ethanol. Samples were identified morphologically using an identification key (Tanaka 1979). Vouchers from Maple Ridge, Burnaby, and Saanichton were deposited in the Beaty Biodiversity Museum at the University of British Columbia (UBC), Vancouver (SEM-UBC-CUL: 0577, 0569, 0570, 1292, 1293).

DNA extraction and PCR

DNA was extracted from the whole body of individual mosquitoes using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) with insect specific protocols. The extracted DNA was used in standard PCR reactions to amplify a region of the cytochrome c oxidase 1 (COI) gene and a region of the NADH dehydrogenase 4 (ND4) gene (Table 1). Both genes are mitochondrially encoded and commonly used in barcoding and phylogeny studies (Zhong et al. 2013, Zielke et al. 2014, Murugan et al. 2016). Each PCR reaction was 25 µl and contained 15 µl nuclease-free water, 7 µl 2X Taq Master Mix (Applied Biological Materials, Richmond, BC), 1 µl forward primer (50 ng/ul), 1 µl reverse primer (50ng/ul), and 1µl DNA template. Thermocycling conditions for CO1 amplification were 94˚ C (5 mins), followed by 35 cycles of 94˚ C (30 sec), 50˚ C (1 min) and 72˚ C (1 min), followed by a 5 min extension at 72˚ C. Thermocycling conditions for ND4 amplifications were 96˚ C (10 mins), followed by 40 cycles of 94˚ C (40 sec), 55˚ C (40 sec) and 72˚ C (1 min), followed by a 10 min extension at 72˚ C. Aliquots of PCR products were size fractionated on a 1.5% agarose gel and quantified using either a Nanodrop 2000c Spectrophotometer (Thermo Scientific, Waltham, MA), or a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA), before being cleaned and sequenced at UBC's Nucleic Acid Protein Services Unit (Vancouver, BC) or through GENEWIZ (South Plainfield, NJ).

Sequencing and alignments

Sequences were trimmed manually using BioEdit (v7.1.11) (Hall 1999) to remove primer regions and submitted to NCBI's Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) to confirm their correspondence to the

Table 1. Primer names, sequences, predicted amplicon sizes, and references for the amplification of the cytochrome oxidase 1 (COI) and NADH dehydrogenase subunit 4 (ND4) genes in *Aedes japonicus*. PMID = PubMed Identification number.

Gene	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Amplicon size	Reference (PMID)
COI	GGT CAA CAA ATC ATA AAG ATA TTG G	TAA ACT TCA GGG TGA CCA AAA AAT CA	~ 617bp	19960671
ND4	CGT AGG AGG AGC AGC TAT ATT	AAG GCT CAT GTT GAA GCT CC	~ 330bp	11296814

appropriate *Ae. japonicus* gene. We downloaded all available *Ae. japonicus* COI and ND4 sequences from GenBank and included corresponding sequences of two outgroup species, *Aedes vexans* and *Aedes togoi*, for phylogenetic analysis. Sequences were aligned using ClustalW (Larkin et al. 2007) in MEGA X (Kumar et al. 2018). All available ND4 sequences aligned well with our samples and were included in the study. Downloaded COI sequences from different regions of the gene (different primers), which had alignment gaps greater than or equal to 50 nucleotides were not included in the analysis. In total, 128 COI and 44 ND4 *Ae. japonicus* sequences were included in this study.

Genetic variability measures

Genetic variability for the COI and ND4 genes of the BC samples was evaluated by finding the number of variable sites (*S*), the average number of nucleotide differences (*k*), and the nucleotide diversity (π) using DnaSP v6.0 (Rozas et al. 2017). The unphased data command in DnaSP v6.0 (Rozas et al. 2017) was used with the haplotype reconstruction function (PHASE method, number of iterations:100, thinning interval:1, and burn-in iterations:100). The outputs of the PHASE reconstructions were used in conducting the Tajima's *D* (Tajima 1989) and Fu's Fs neutrality tests (Fu 1997) at an α=0.05 using Arlequin v3.5.2.2 (Excoffier and Lischer 2010). Analysis of molecular variance (AMOVA) (Excoffier et al. 2010) was performed using Arlequin v3.5.2.2 (Excoffier and Lischer 2010) with 1000 permutations to determine the genetic differentiation among and within the populations. Pairwise F_{ST} (fixation index) values with 1000 permutations were also obtained for the COI and ND4 genes of the BC samples using Arlequin v3.5.2.2 (Excoffier and Lischer 2010). Because we only had single specimens from Everett, Washington and Hamilton, Ontario, the same analysis could not be carried out for the samples from these locations.

Phylogenetic analysis

All phylogenetic analyses were conducted using MEGA X (Kumar et al. 2018). We began by finding the optimum substitution model using the best fit model test. A Tamura-Nei (TN93) model (equal transversion but variable transition rates) using a discrete Gamma distribution (+G) was determined to be the optimum model for the COI sequences as it had the lowest Bayesian Information Criterion (BIC) score out of 24 different models. For the ND4 tree, the Hasegawa-Kishino-Yano (HKY) model (one transition and one transversion rate) using a discrete Gamma distribution (+G) was determined as the optimum model. The maximum likelihood (ML) parsimony tree for each set of sequences was constructed using a bootstrap of 1,000 replications. The COI tree compared 130 different sequences, including two outgroups, each consisting of 617 nucleotides. The ND4 tree compared 46 different sequences, including two outgroups, each consisting of 330 base pairs. Phylogenetic trees were visualized and annotated using FigTree v1.4.3 (http://tree.Bio.Ed.Ac.Uk/software/figtree/).

Haplotype network analysis

Haplotype networks were constructed in PopART v.1.7 (Leigh and Bryant 2015) using the Templeton, Crandall, and Sing (TCS) method (Clement et al. 2000). Sequences with unknown nucleotides (Ns), outgroups and the *Ae. japonicus* subspecies sequences *(Ae. j. yayaemensis, Ae. j. amamiensis,* and *Ae. j. shintienensis)* were excluded from the haplotype networks to present the relationship between *Ae. j. japonicus* sequences. The COI and ND4 TCS networks were constructed from 122 and 34 sequences, respectively, which were then grouped into haplotypes. Haplotype frequencies were represented visually via location-based pie charts.

RESULTS

Sequencing and alignments

All sequences obtained confirmed the successful PCR amplification of *Ae. j. japonicus* cytochrome oxidase 1 (COI) and NADH dehydrogenase 4 (ND4) genes. BLAST analysis showed 99-100% identity and e-values near or equal to zero for all sequences. The sequence alignments were devoid of introns, indels, and pseudogenes. A total of 617 nucleotide sites and 330 nucleotide sites were analyzed for the COI and ND4 genes, respectively.

Genetic variability measures

Genetic diversity indices were calculated for the three BC populations (Table 2). Average nucleotide differences (*k*) were low within and between the three populations for both COI and ND4 sequences ranging from 1.048-2.000 and 1.286-1.530, respectively. Similarly, the nucleotide diversity (π) measures were below 0.01 for all populations, indicating very low genetic variability among *Ae. j. japonicus* collected

Table 2. Genetic diversity indices and demographic statistics of *Aedes japonicus japonicus* populations in British Columbia. ND4 = NADH dehydrogenase 4, COI = cytochrome c oxidase 1, *N* = number of sequences, *S* = number of variable sites, *k* = average number of nucleotide differences, and π = nucleotide diversity.

Location	Gene	*N*	*S*	*k*	π	Tajima's D	Tajima's D P value	Fu's Fs	Fu's Fs P value
Burnaby 49.277, -122.915	COI	6	3	1.467	0.00238	0.60031	0.72	-1.07189	0.08
	ND4	3	4	1.530	0.00454	0.54631	0.71	0.30438	0.58
Maple Ridge 49.219, -122.564	COI	7	3	1.048	0.00170	-0.65405	0.31	0.10980	0.46
	ND4	6	4	1.484	0.00440	0.59112	0.73	0.46279	0.62
Saanichton 48.558, -123.365	COI	4	4	2.000	0.00324	-0.78012	0.19	2.19722	0.82
	ND4	4	3	1.286	0.00382	0.45766	0.74	2.46884	0.89
All BC	COI	17	6	1.750	0.00284	-0.04680	0.53	-1.98317	0.07
	ND4	17	5	1.629	0.00483	0.87867	0.81	0.86306	0.72

in BC. Higher nucleotide diversity was observed in the ND4 gene compared to the COI gene in all three populations and consequently, in the All BC samples group.

Tajima's D statistic showed negative values for most COI groups, and positive values for ND4 groups. Only Burnaby samples had positive Tajima's D statistic values for both genes (Table 2). None of these differences were found to be statistically significant ($p \geq 0.05$). The results of the second neutrality test (Fu's F) yielded a negative result for the Burnaby COI subgroup and the combined All BC COI group but positive values for all other subgroups (Table 2). Again, none of these differences were statistically significant ($p \geq 0.05$). Statistically, the null hypothesis of a constant size population under the neutral model (mutation-drift equilibrium) could not be rejected (Tajima 1989, Fu 1997).

The AMOVA of the COI and ND4 dataset of *Ae. j. japonicus* collected in Burnaby, Maple Ridge, and Saanichton BC indicated that most of the molecular variation was observed within the populations (COI: 73.53%, ND4: 84.88%) and less among the populations (COI: 26.47%, ND4: 15.12%) ($p<0.05$) (Table 3). Pairwise fixation index (F_{ST}) values of both genes (COI and ND4) for the three populations are presented in Table 4. The values ranged from 0 (no genetic subdivision between the two populations) to 0.419 (an F_{ST} value of 1 indicates a complete genetic differentiation between the two populations). The results from both genes indicated no differentiation between the Maple Ridge and Saanichton populations. The highest F_{ST} value was observed in the COI gene of the Burnaby and Maple Ridge populations.

Phylogenetic analysis

Molecular phylogenetic relationships among samples were inferred using Maximum Likelihood (ML) methods for both genes. The ML tree for the ND4 gene was not informative in inferring geographic relationships because all the BC *Ae. j. japonicus* samples were observed within the same clade alongside specimens from the United States and Europe (Figure 1) with low bootstrap values (17.4%). This clade was clearly distinct from the other subspecies of *Ae. japonicus (Ae. j. yayaemensis, Ae. j. amamiensis,* and *Ae. j. shintienensis)* as described previously (Cameron et al. 2010), confirming that our BC specimens are indeed *Ae. j. japonicus*. The ML tree for the COI gene was more informative in inferring relationships between our samples and those from around the world (Figure 2). Again, as expected, a clear separation in clades is observed between *Aedes j. japonicus* and other *Ae. japonicus* subspecies. The *Ae. j. japonicus* samples from China form a tight, distinct clade, separate from all other samples with a high bootstrap value (96.3%), although more samples from China are needed to support this relationship. In analyzing the remaining clades, we noticed multi-continental groupings. The largest clade had samples from six different countries (Canada, Germany, Switzerland, United States, Japan, and the Netherlands). In other cases, there were distinct national clades with bootstrap values greater than 60%.

Haplotype network analysis

The genealogical relationships of the haplotypes were constructed based on COI and ND4 sequences using the TCS method in PopART v.1.7 (Figures 3 and 4), differentiating haplotypes based on single nucleotide changes. The COI network shows the lack of geographic distinctiveness as Haplotype 1 (H1) was found on multiple continents . H1 was the most common among the COI sequences with 40.16% (49/122) of total COI samples sharing this sequence. The COI sequences from the BC samples were separated into H1, H3, H6, H15, and H18. The Washington sample was H1 and the Ontario sample was H9. The most divergent COI sequences from H1 are samples from Asia that represent H22, H23, and H24. Fewer ND4 sequences were publicly available and, as such, the ND4 network contains fewer haplotypes and less information, although it follows similar trends to the COI haplotype network. Again, in the ND4 network, the most common haplotype was the H1 haplotype, containing samples from North America and Europe. The haplotype diversity is higher in COI European samples, and in ND4 USA samples, although this is likely due to a sampling bias in these locations. In both analyses, Asian samples contained unique haplotypes that were not present in other locations (ND4: H7, COI: H5, H14, H19, H20, H21, H22, H23, H24). These results indicate that the ND4 marker, alone, may not be suitable in determining species-level genetic relationships

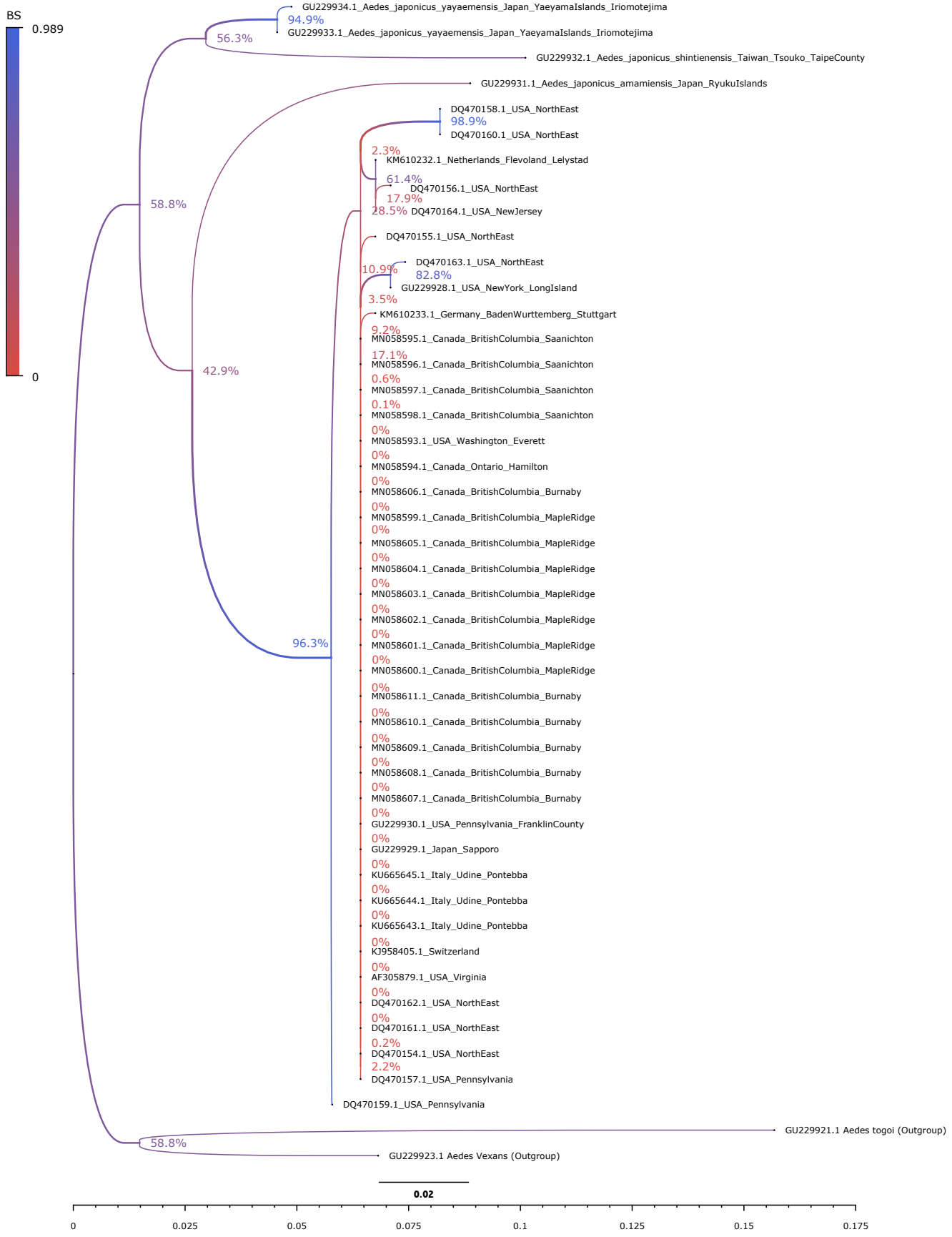

Figure 1. Maximum-likelihood (ML) tree using aligned ND4 sequences. Branch names show the GenBank sequence identifier and geographic location of samples. Bootstrap (BS) values are shown on branches as percentages. The scale bar represents genetic change by the number of nucleotide substitutions per site.

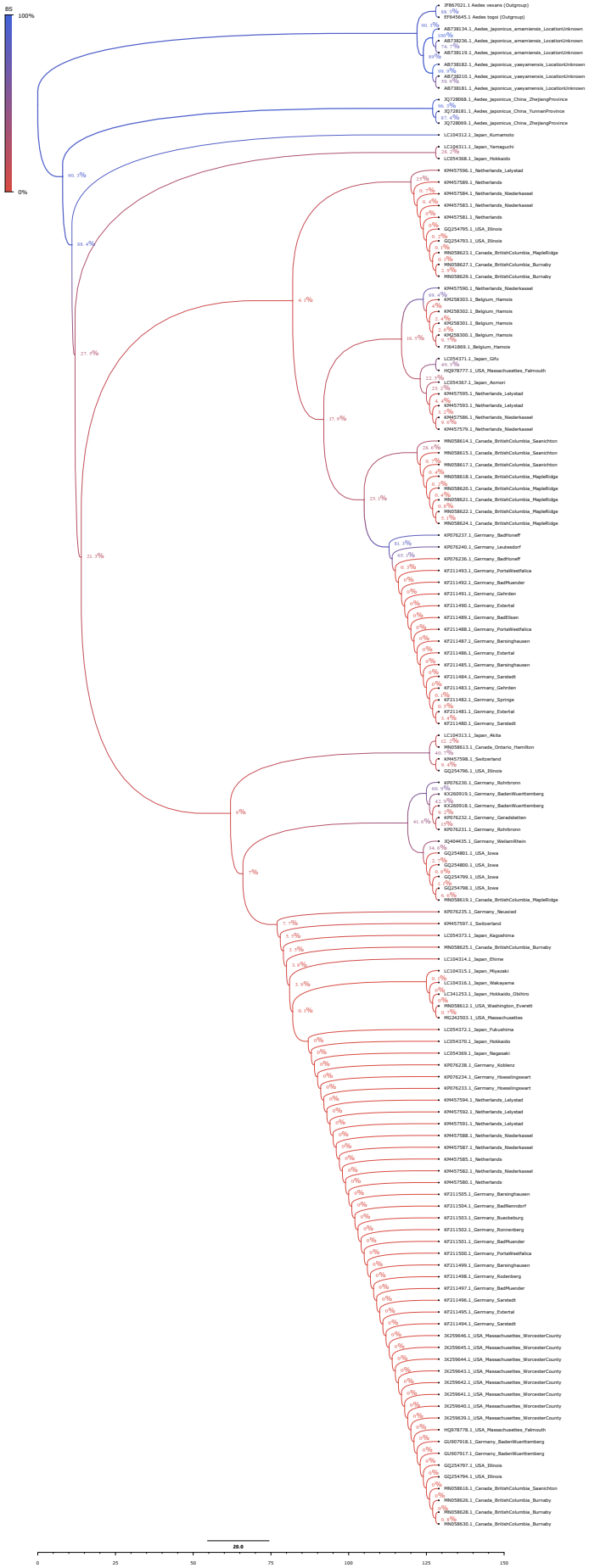

Figure 2. Maximum-likelihood (ML) tree using aligned COI sequences. Branch names show the GenBank sequence identifier and geographic location of samples. Bootstrap (BS) values are shown on branches as percentages. The scale bar represents genetic change by the number of nucleotide substitutions per site.

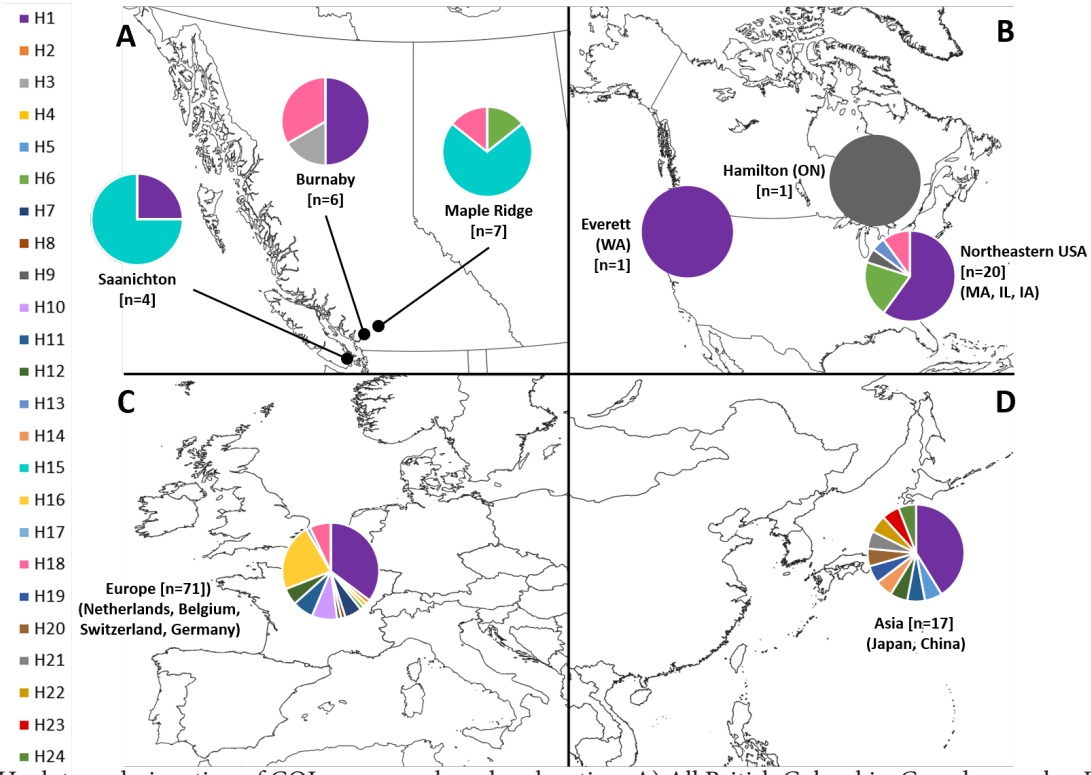

Figure 3. Haplotype designation of COI sequences based on location. A) All British Columbia, Canada samples, B) Non-British Columbia North American samples (MA = Massachusetts, IL = Illinois, IA = Iowa, WA = Washington, ON = Ontario, Canada, C) European Samples, and D) Asian samples.

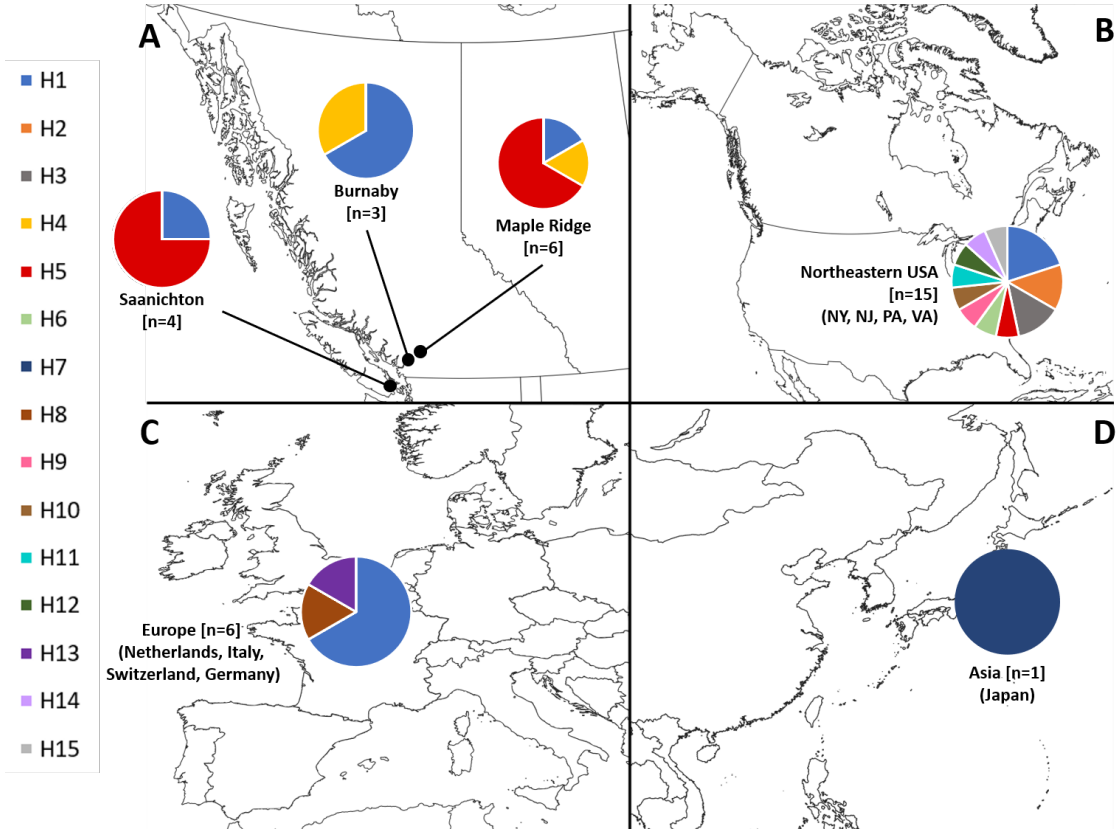

Figure 4. Haplotype designation of ND4 sequences based on location. A) All British Columbia, Canada samples, B) Non-British Columbia North American samples (NY = New York, NJ = New Jersey, PA = Pennsylvania, VA = Virginia), C) European Samples, and D) Asian samples.

Table 3. Analysis of Molecular Variance (AMOVA) among and within populations of the cytochrome oxidase 1 (COI) and NADH dehydrogenase subunit 4 (ND4) genes in *Aedes japonicus japonicus* in British Columbia. df = Degrees of freedom.

Gene	Source of variation	df	Sum of squares	Variance components	Percentage of variation	P value
COI	Among populations	2	4.190	0.25221	26.47	P < 0.05
	Within populations	14	9.810	0.70068	73.53	P < 0.05
	Total	16	14.000	0.95289		
ND4	Among populations	2	4.323	0.12964	15.12	P < 0.05
	Within populations	31	22.560	0.72773	84.88	P < 0.05
	Total	33	26.882	0.85737		

in *Ae. j. japonicus* due to low sequence diversity. The TCS method has been used extensively to infer population level genetic relationships when divergences are low (Clement et al. 2000). Our results indicate that using COI and ND4 markers alone does not allow for accurate geographic determination of newly arrived *Ae. j. japonicus* populations.

DISCUSSION

We found low nucleotide differences (*k*) and low nucleotide diversity (π) for both COI and ND4 (k ranging from 1.048-2.000 and 1.286-1.530, respectively, π <0.01 for all populations) in all our BC samples. Comparably low indices of nucleotide diversity have been observed in other *Aedes sp.* (Naim et al. 2020, Cevallos et al. 2020). This lack of genetic variation via COI and ND4 may suggest that our BC samples are the result of a population bottleneck (Nei et al. 1975), and as such, are quite closely related. The construction of haplotype networks that relied on ND4 and COI sequences showed three different ND4 haplotypes and five different COI haplotypes in our BC samples. Three of these haplotypes were found exclusively in BC samples (ND4 H4 in Burnaby and Maple Ridge, COI H3 in Burnaby, and COI H15 in Saanichton and Maple Ridge). The remaining haplotypes were shared among different BC locations, as well other North American samples (ND4 H5), North American and European samples (ND4 H1, COI H18), and North American, European, and Asian samples (COI H1). These data could indicate i) multiple separate introduction events for the Burnaby, Maple Ridge and Saanichton samples, ii) potentially more permanent populations in each of these three locations (as evidenced by the haplotypes in each location), or iii) introduction events from another unsampled location. Our results may be affected by the ability of *Ae. japonicus* to undergo diapause, which has allowed species such as *Aedes albopictus* to colonize more disperse areas, without the need to colonize intermediate locations (Schmidt et al. 2020). Due to the high haplotype diversity throughout North America and Europe, it is not clear where, precisely, the BC introductions originated. As the Asian haplotypes were mostly in a different clade for both ND4 and COI sequences, it is unlikely that BC samples originated from Asia.

Table 4. Matrix of pairwise Fixation Index (F_{st}) values between *Aedes japonicus japonicus* populations in British Columbia. Values for the COI gene are in the Lower diagonal and values for the ND4 gene in the upper diagonal. *$P < 0.05$.

Location	Burnaby	Maple Ridge	Saanichton
Burnaby	-	0.214*	0.226
Maple Ridge	0.419*	-	0
Saanichton	0.245	0	-

These genetic diversity trends were mirrored by our phylogenetic analyses. The ND4 gene cannot effectively show intra-subspecies phylogenetic relationships for *Ae. j. japonicus* using our samples but can be useful in inferring relationships between *Aedes* species as well as genetically distinct *Ae. japonicus* subspecies. In our COI trees, all the BC *Ae. j. japonicus* samples were in the same clade as specimens from the United States and Europe. Furthermore, we noticed low inter-clade BC values within *Ae. j. japonicus* samples, suggesting that the *Ae. j. japonicus* from BC in this study may not have undergone sufficient geographic isolation that would lead to discernable COI and ND4 genetic differentiation. Additionally, our results indicate there is more genetic variation within BC *Ae. japonicus* populations than between these populations. However, the F_{st} values obtained in this study demonstrate a low amount of gene flow between these populations, indicating *Ae. japonicus* exists, at least currently, in somewhat discrete populations in BC.

It is also possible that these populations are genetically distinct, but the two genes (COI and ND4) we used to study *Ae. j. japonicus* population genetics were not truly representative of the populations' genetic structures. Admittedly, sample sizes in our study were lower than optimum, constrained by the number of specimens available, causing the power of the

analysis to be relatively low. Nevertheless, our findings are similar to conclusions drawn by Zielke et al. (2015) and are in line with expected results of low genetic diversity from situations representing new introductions and founder events (Tsutsui et al. 2000).

An alternate approach would be to use microsatellite analyses, as utilized by Zielke et al. (2015) to differentiate *Ae. japonicus* in Europe. Ideally, high resolution, genome-wide SNP markers would be used to create a genetic signature of these populations to pinpoint a specific introduction locale. The mtDNA techniques we employed allow for a broad view of a species' origin of introduction, but lack the wide range and high number of analysis sites that SNP based protocols employ (Gloria-Soria et al. 2018, Schmidt et al. 2019). However, a reference panel is needed for these analyses, and currently, there are no globally available SNP *Ae. japonicus* datasets. Furthermore, these techniques are commonly applied to study *Ae. aegypti* population structure and are very fitting based on the high level of genetic differentiation within the species. Such precision is harder to achieve from species with lower genetic differentiation than *Ae. aegypti* (Schmidt et al. 2020), which is likely the case for *Ae. japonicus*. Studies can combine multiple mitochondrial loci (COI, COII, and ND4) in addition to nuclear DNA as genetic markers for population genetic analyses, but these approaches face complications due to combined trees requiring multilocus sequences that are not only from the same region of collection but also from the same individual specimen. Therefore, such an analysis is more conceivable when the sequencing and analysis are conducted within the same experiment as opposed to studies that rely on globally contributed datasets such as GenBank or BOLD (Barcode of Life).

Aedes j. japonicus is well-known to capitalize on human transportation to spread over long distances (Egizi et al. 2016), and one possible explanation for the low genetic diversity in our samples is that the population of *Ae. j. japonicus* in western Canada originated from a small number of individuals introduced via human-assisted transport. For example, eggs or larvae transported in tires tend to be few in number and can go undetected from one destination to the next (Laird et al. 1994). This would explain the low genetic diversity in BC populations if mosquitoes were imported from a single region as opposed to multiple introductions of genetically diverse individuals. Alternatively, the low diversity could be a product of multiple introductions by genetically similar individuals. Invasion of new habitats can cause new environmental challenges and pressures on populations; however, the three BC collection sites, (Maple Ridge, Burnaby, and Saanich), do not represent drastically different environmental conditions that would increase genetic diversity. Diverse environments can cause an increase in species-level diversity when populations have been present for longer periods and isolated from gene flow (Amos and Harwood 1998), both situations that are unlikely in the populations that we studied.

Phylogeography compares genetic variation within alleles to formulate population inferences within a country (Fonseca et al. 2010, Versteirt et al. 2015) or on a larger scale by comparing different regions in many different countries (Cameron et al. 2010, Zielke et al. 2014, 2015). The latter approach requires large-scale sampling and sequencing studies with available databases that link taxonomic and genetic information with accurate geographic information. Our data cannot pinpoint the source location of the populations introduced into BC. Our data suggest that the *Ae. j. japonicus* samples collected in BC are most similar to those from Germany, the Netherlands, and the United States (Iowa). Despite significant and frequent commercial shipping from Asia to the Vancouver region, the BC samples do not align well with samples from China or Japan, instead suggesting introductions from Europe directly or from eastern North America. This introduction pattern is concerning given the recent detection of overwintering *Ae. albopictus* in Eastern Canada (Giordano et al. 2020) and the suitable climate predicted for this species in BC (Kamal *et al.* 2018). Future efforts should focus on detecting and preventing new *Ae. j. japonicus* introductions, recognizing that using mtDNA approaches similar to those used here are unable to narrow down the source of the introduction, and as such, higher resolution markers such as genome-wide SNPs should be employed in future studies. Furthermore, this study highlights the need for consistent and frequent mosquito surveillance in order to flag potential invasive species introductions and to monitor local biodiversity.

DATA AVAILABILITY

All sequences from samples generated in this study are publicly available through the NCBI GenBank database: Accession numbers MN058593-MN058630.

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