Population Structure and Gene Flow in the White Pine Weevil, *Pissodes strobi* (Coleoptera: Curculionidae)

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ABSTRACT The white pine weevil, *Pissodes strobi* (Peck), is a major source of damage to young spruce and pine trees across North America. The species contains a substantial amount of genetic, morphological, and behavioral variation, and identification of patterns of genetic variation on a broad geographic scale may contribute toward more effective pest management strategies. To estimate maternal gene flow and examine the genetic structure of *P. strobi* we sequenced an 826-bp fragment of the mitochondrial DNA (mtDNA) COI gene in 130 individuals from 11 locations across Canada. Nested clade analysis of 36 haplotypes yielded three patterns of genetic structuring that are inferred as due primarily to restricted gene flow and contiguous range expansion, with one case of long-distance colonization. Analysis of molecular variance analysis also showed significant genetic structuring and restricted gene flow among regional populations. Eastern and western populations were divergent, as were the four populations surveyed in British Columbia. Findings were comparable with those of previous studies based on allozyme or randomly amplified polymorphic DNA data, although population differentiation was greater in mtDNA. Detection of such genetic structure may be important for control programs, because other studies have shown that the conifer hosts of *P. strobi* provide geographically structured variation in resistance to damage.

KEY WORDS *Pissodes strobi*, gene flow, population structure, nested clade analysis

THE WHITE PINE WEEVIL, *Pissodes strobi* (Peck), is native to North America, where it is a serious pest of regenerating pine and spruce stands. Adult weevils oviposit in the previous year's terminal leader, and developing larvae feed on phloem and move down the stem, destroying the growth of the previous 2–4 yr (Drouin and Langor 1991). After pupation, adults emerge by boring out of the stem, leaving holes that may facilitate entry of tree pathogens (Ostrander and Foster 1957). Repeated yearly attacks occur commonly and can result in significant economic losses for the lumber industry and in ornamental trees due to loss of growth, stem deformation, and cull (Brace 1972, Gross 1985). A great deal of effort has been invested in studying this pest and in developing management options (Langor 1998).

P. strobi is transcontinental in North America, ranging from the southern part of the Yukon and Northwest Territories to Oregon, Utah, and Colorado in the west, and from northern Quebec to North Carolina and northern Mississippi in the east (Langor and Sperling 1995). The species does not disperse long distances (Tomlin et al. 1997) and exists in a series of distinct populations across its range (Lewis et al. 2001). *P. strobi* attacks many species of *Picea* and *Pinus* within its range and host use varies regionally. In eastern North America, *P. strobi* is found mainly on pines but is also common on spruces; however, in the Great Plains and Canadian prairies and westward it attacks primarily spruces and is found rarely on pines (Humble et al. 1994, Phillips and Lanier 2000).

In species with broad geographic and host ranges it is not unusual to find considerable genetic variation (Avise 1994). In his revision of *Pissodes*, Hopkins (1911) recognized *P. strobi* as a species from pines and spruces in eastern North America. Although he acknowledged that terminal-infesting weevils on spruce in western North America were closely related to *P. strobe*, he concluded that there were sufficient differences in morphology and host association to warrant description of two new species, *P. sitchensis* (Bong.) Carr on Sitka spruce and *P. engelmanni* (Parry) on Engelmann spruce. These two species were subsequently synonymized with *P. strobi* based on cytogenetic evidence, successful cross-breeding, and similar ecology and behavior (Smith and Sugden 1969), and later corroborated with allozyme, mitochondrial DNA (mtDNA), and morphological studies (Phillips and Lanier 2000; Langor and Sperling 1995, 1997; Williams and Langor 2002a,b). However, independently of taxonomic rank, it is clear that much genetic variation exists throughout the geographic and host range of *P. strobi.* Such variation may have significant management implications for this economically important

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Table 1. Collection localities of sampled *P. strobi*

Locality	Collection locality	Latitude	Longitude	No. of specimens	Population group	Host
1	Kitimat, BC	54.081° N	128.693° W	12	West 1	*Picea sitchensis*
2	Benson River, BC	50.607° N	127.602° W	12	West 2	*Picea sitchensis*
3	Sechelt, BC	49.495° N	123.787° W	12	West 2	*Picea sitchensis*
4	Fort Nelson, BC	58.825° N	122.775° W	12	West 3	*Picea glauca*
5	Vernon, BC	50.325° N	119.275° W	12	West 4	*Picea glauca*
6	Bertwell, SK	50.866° N	101.893° W	12	Central	*Picea glauca*
7	Sandilands, MB	49.375° N	95.575° W	12	Central	*Pinus banksiana*
8	Massey, ON	46.332° N	82.200° W	11	East	*Pinus banksiana*
9	Launay, QC	48.459° N	78.065° W	12	East	*Picea glauca*
10	Victoria County, NS	44.337° N	65.266° W	11	East	*Pinus strobus*
11	Delaney, NS	45.263° N	62.053° W	12	East	*Picea abies*

ON, ontario; QC, Quebec; SK, Saskatchewan.

species and justifies continuing effort to describe patterns of genetic variation.

mtDNA has been used to elucidate population structure, taxonomy, and phylogenetic relationships in *Pissodes* (Boyce et al. 1994; Langor and Sperling 1995, 1997). The advantages of mtDNA are that it has relatively rapid evolutionary rates and so differences between populations that diverged a relatively short time ago may be detected. There is also little within-individual variation in coding sequences, so individuals are functionally haploid in coding regions due to maternal inheritance (although Boyce et al. (1994) found within individual variation in the AT-rich region). Thus, mtDNA serves as a useful marker of maternal gene flow, which is of particular interest in the context of host race formation. Boyce et al. (1994) examined restriction site variation in 11 populations of *P. strobi* throughout most of its range and found 41 haplotypes; however, insufficient information was given to provide insight into geographic variation. In a further sampling of *P. strobi* that was more limited geographically (nine populations, mainly from western Canada), Langor and Sperling (1995) found 10 haplotypes from restriction sites in a 1585-bp fragment of mtDNA. One haplotype was widespread throughout most of the sampled range and the remainder were more localized, usually near the margins of the range of the species; however, the small number of individuals ($N = 55$) examined in that study and the limited number of restriction sites surveyed were insufficient to provide a good estimate of gene flow in *P. strobi.*

More recent studies of genetic variation in *P. strobi*, based on randomly amplified polymorphic DNA (RAPD) markers and allozymes, have shown the presence of three genetically distinct groups of populations of *P. strobe:* one including populations from the south coast of British Columbia (BC) and Vancouver Island, one from the north coast of BC, and one including all populations from the interior of BC and east of the Continental Divide (Lewis et al. 2000, 2001). Allozyme analyses have shown that genetic diversity decreases from northeastern North America to the Pacific Coast, as measured by number of alleles per locus, percentage of polymorphic loci and percent heterozygosity (Phillips and Lanier 2000; Lewis et al. 2000), and this pattern may indicate range expansion of *P. strobi* from east to west. In Pacific Coast populations, the absence of alleles that are present to common in more eastern populations, and the high frequency of alleles normally rare in eastern populations, also suggest westward range expansion through a series of founder events.

To build on previous work and describe large-scale population genetic structure in *P. strobi* across its range, as well as to provide quantitative estimates of maternal gene flow that provide independent data to test inferences about gene flow based on allozyme and RAPD data, we assessed sequence variation among *P. strobi* populations throughout its range in Canada. We amplified and sequenced an 826-bp fragment of mtDNA from the cytochrome oxidase I region because this region had demonstrated unusually high sequence divergence in *Pissodes* relative to allozyme variation (Langor and Sperling 1997). Such sequences provide phylogenetic information that can be used effectively in nested clade analysis to identify regions with genetically distinct populations, as well as to uncover historical patterns responsible for current genetic structure.

Materials and Methods

Individuals of *P. strobi* were collected from 11 localities across Canada (Table 1). Ten of these localities (populations) were also used by Lewis et al. (2000) for allozyme work; the 11th locality was Victoria County, Nova Scotia. Weevil-infested terminals were clipped in July 1992 in Victoria County, and in July 1996 at the other 10 localities and returned to the laboratory where terminals were individually reared. Emerged adults were frozen at −25°C before DNA extraction. Within populations, each sequenced weevil was reared from a different terminal, to ensure that distinct parental lineages were sampled. The abdomens and elytra were removed from each specimen before DNA extraction, preserved in 95% ethanol, and placed as vouchers in the reference collection at the Northern Forestry Centre of the Canadian Forest Service (Edmonton, Alberta). Specimens were identified based on a combination of morphological and behavioral characters, as well as host plant damage characteristics.

Mitochondrial DNA was extracted from the head, thorax, and legs of 130 individuals by using the phenol/chloroform method, as described previously (Langor and Sperling 1994). Extracted DNA was stored at −17°C, before amplification via polymerase chain reaction (PCR). An 826-bp fragment of the cytochrome oxidase I gene was amplified, corresponding to base pairs 2184–3009 of *Drosophila yakuba* (Burla) (Clary and Wolstenholme 1985). mtDNA was amplified in a 50-μl reaction solution by using 35 μl of double-distilled (Millipore Corporation, Billerica, MA) water, 5 μl of 25 mM $MgCl_2$ (QIAGEN, Valencia, CA), 5 μl of 10× buffer (QIAGEN), 1 μl of 10 mM dNTPs (Roche Diagnostics, Indianapolis, IN), 1 μl each of two 5 pM/μl heterologous primers C1-J-2183 (5′-CAACATTTATTTTGATTTTTTGG) and TL2-N-3014 (5′TCCAATGCACTAATCTGCC ATATTA) (Simon et al. 1994), and 1 μl of extracted DNA. Between 0.5 and 1.0 U of DNA *Taq* polymerase was added during the annealing step of the first cycle. PCR amplification was performed using a T-Gradient PCR thermal cycler (Biometra, Göttingen, Germany). The PCR consisted of 35 cycles, with the following program: denaturation for 1 min at 94°C, annealing for 1 min at 45°C, and extension for 1.5 min at 72°C. PCR products were cleaned using the QIAquik PCR purification kit (QIAGEN).

Both the forward and reverse strands were sequenced using two internal primers C1-N-2659 (5′-ACTAATCCTGTGAATAAAGG) (modified from Simon et al. 1994) and C1-J-2495 (5′-CCTCCTCTTT ATGATCAAT TGG) (modified from Cognato and Sperling 2000) and the primers used for PCR amplification. Sequencing reactions used 1 μl of purified PCR product, 1 μl of BigDye terminator cycle sequencing mix, and 4 μl of 2.5× buffer (Applied Biosystems, Foster City, CA), 3.5 μl of double-distilled (Millipore Corporation) water, and 0.5 μl of one of the four primers mentioned above. The thermal cycler program was as follows: one cycle at 96°C for 2 min, 25 cycles at 96°C for 30 s, 50°C for 30 s, and 60°C for 4 min. Sequencing products were then cleaned using Sephadex G-50 fine columns (Amersham Biosciences Inc., Piscataway, NJ) and visualized on an ABI 377 automated DNA sequencer (Applied Biosystems). Contigs were produced using Sequencher 4.1 (Gene Codes Corporation, 2001). Because there were no insertions or deletions, the sequences from different individuals were aligned by eye by using PAUP version 4.0 beta nine (Swofford 2002). Parsimony analysis and bootstrapping were also carried out with PAUP (Swofford 2002) by using sequence from *P. terminalis* and *P. nemorensis* as outgroups (GenBank #U77980 and U77981, respectively).

Two programs were used to perform nested clade analysis. First, TCS 1.13 (Clement et al. 2001) was used to estimate a network of relationships among haplotypes. This program was used to obtain a 95% confidence limit for parsimony (Templeton et al. 1995) as well as to construct a cladograph that showed the nested structure of the haplotypes. Once each haplotype's position within specific clades was determined, GeoDis 2.0 (Posada and Templeton 2000) was used to calculate distances between populations where haplotypes or clades occurred. Geographic distance was measured using latitude and longitude in decimal degrees (Table 1).

Arlequin 2.0 (Schneider et al. 2000) was used to perform an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) and to estimate F_{ST} values for genetic variation between populations and groups of populations of *P. strobi.* AMOVA also was used to examine recent geographic structure, especially to detect isolation by geographic distance. Localities were grouped into six regions for AMOVA, one in the east, four in the west, and one in central Canada, based on genetic groupings by Lewis et al. (2000). AMOVA results also were calculated for two other groupings to verify whether this study confirms previous groupings based on allozymes (Lewis et al. 2001) and RAPDs (Lewis et al. 2000). First, the eastern and central regions together were compared with the western populations (location categories are indicated in Table 1). Second, only the western populations were compared with each other to examine structuring among western populations.

Results

Thirty-six haplotypes were found among the 130 specimens sequenced (GenBank accession nos. AY472043–AY472078), including 28 at single localities, seven at two localities, and one at three localities (Fig. 1). The *P. strobi* sequence previously deposited in GenBank by Langor and Sperling (1997) (GenBank #U77976) gave a 37th haplotype, but it is not considered in nested clade analysis because it represents a single individual in its population. Haplotype h7 was the one most frequently observed, in 12 individuals and three populations. Except for haplotype 1, which was found seven times in one locality, all other haplotypes were found five or less times in one or two localities (Fig. 1). Sequence divergence among haplotypes ranged from 0.12 to 2.54%, with an overall nucleotide diversity for the 36 haplotypes of 0.86%.

Parsimony analysis (Fig. 1) did not resolve relationships among haplotypes very well, and bootstrap values were not high except at the base of the ingroup. Haplotypes found in eastern populations tended to be more basal than in western populations, but this difference was not consistent.

Haplotype network construction by TCS 1.13 (Clement et al. 2001) yielded a single network with a maximum of 22 mutational steps between any two haplotypes (Fig. 2). The haplotype network had two main clades, 4-1, and 4-2. There were 24 one-step clades, nine two-step clades, and four three-step clades.

Nested contingency analysis from GeoDis 2.0 (Posada and Templeton 2000) showed 15 significant associations between clades and sampling locations (Table 2). Although there was little significant geographic association at the one-step clade level (only

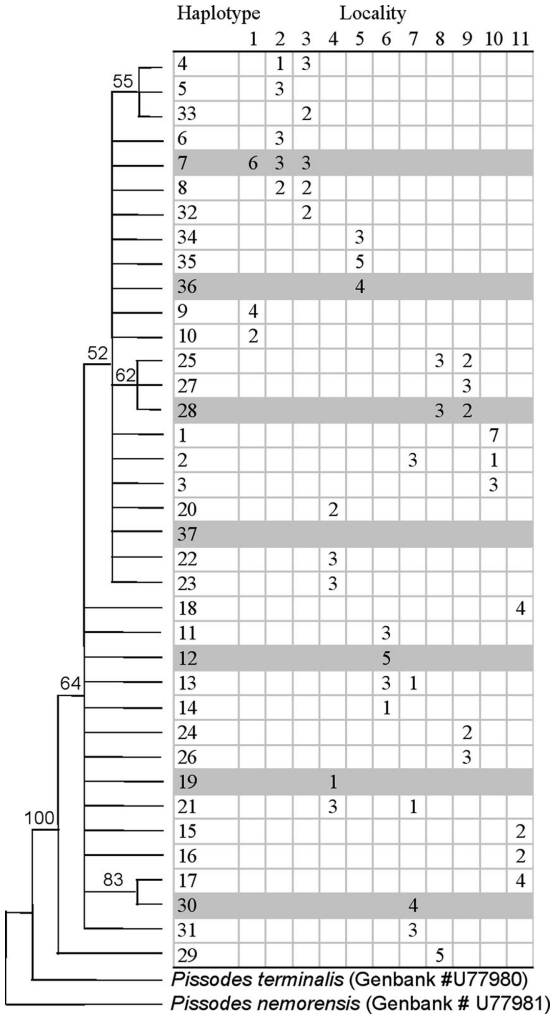

Fig. 1. Bootstrap consensus tree from parsimony analysis of mtDNA of *P. strobi*, showing bootstrap support above branches and geographic distribution of haplotypes. Localities correspond to those in Table 1.

1–15), all higher-level clades, except 2-1 and 3-1, showed significant levels of geographical association.

Based on both the inference key given in Templeton et al. (1995) and the geographical distribution of clades, nested clade distance analysis yielded inferred patterns at all clade levels (Table 3). Three inferred patterns were evident in five nested clades, representing three hierarchical levels. Restricted gene flow due to isolation by distance was the most common explanation for the patterns observed. In three nested clades, representing two hierarchical levels, contiguous range expansion is inferred, and in one clade, long-distance colonization is inferred.

AMOVA detected significant structuring between populations in the eastern, central, and western regions of Canada, with almost 21% of variation caused by among-region sources (Table 4). AMOVA also detected significant structuring between eastern/Central regions and the western region, with almost 17% of variation occurring between these two groups. Fine genetic structuring was detected in only the western region populations, with >41% of variation occurring between the different population groups in the west. Significant F_{ST} values from all three analyses indicate overall structuring among populations. Together, AMOVA analyses suggest that restricted gene flow is a result of large geographic distances separating populations.

Discussion

Sequence Divergence and Parsimony Analysis. The 36 distinct haplotypes found for *P. strobi* in this study are 4 times the number found in a previous study of restriction site variation (Langor and Sperling 1995). This increase is not surprising because we sampled a greater number of nucleotides, used larger sample sizes, and sampled more populations separated by larger geographic distances. Intraspecific sequence and nucleotide diversity also were very high compared with other species of insects, including Lepidoptera, Coleoptera, Diptera, and Hemiptera (Langor and Sperling 1997, Althoff and Pellmyr 2002), which would help to account for the high number of haplotypes. Because no single haplotype was found in more than three populations, it was not possible to identify an ancestral haplotype.

Poor resolution in parsimony analysis and low bootstrap values are caused by low variability between many haplotypes, even if overall sequence divergence is high. This low variability between some haplotypes is likely due to the recent formation of haplotypes and results in a star phylogeny in parsimony analysis.

Population Structure and Gene Flow. One previous study of *P. strobi* variation in western Canada showed haplotype variation where one widespread haplotype was found throughout most populations, whereas other haplotypes were localized to one or two populations near the margins of the species range (Langor and Sperling 1995). This was interpreted to indicate that some gene flow occurred historically between populations. However, the predominant pattern we saw in our study more closely resembled that found by Boyce et al. (1994), wherein haplotypes were restricted to only a few populations. The significant association of many haplotypes and clades with specific populations, as shown by nested contingency analysis, nested clade analysis, and AMOVA, is indicative of restricted gene flow among many populations, or at least between populations that are geographically distant from each other.

AMOVA analysis also was performed on a dataset of restriction site variation in *P. strobi*, by using the five restriction endonucleases reported by Langor and Sperling (1995) and the same 130 samples used in the current study (unpublished data). We obtained results very similar to those obtained in the current study, showing genetic structure between western, Central, and eastern regions, as well as the different populations in the west.

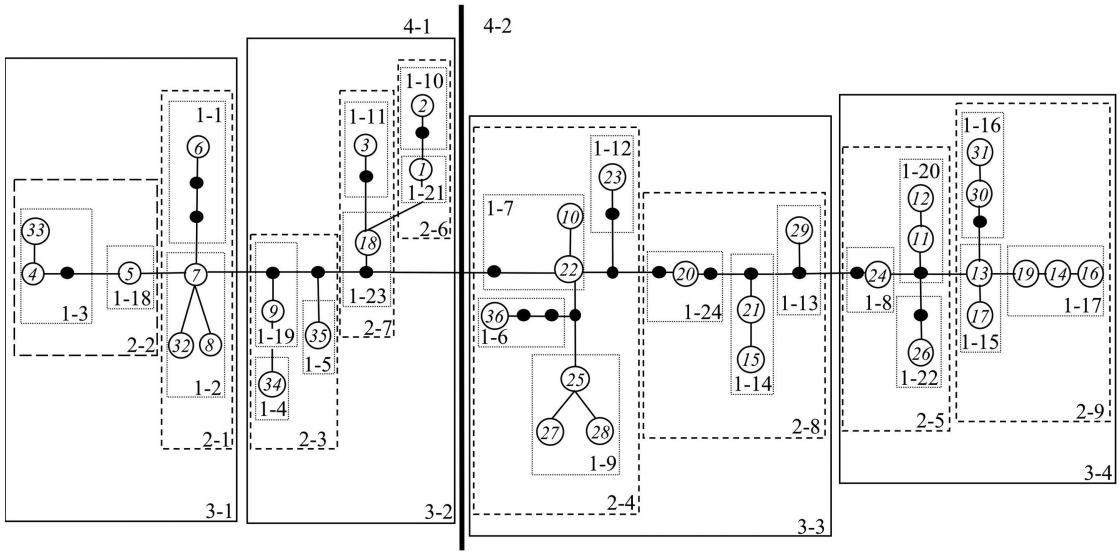

Fig. 2. Haplotype network and nested clade design of the 36 distinct haplotypes detected in the current study of *P. strobi*. Haplotypes are represented by numbers in circles. Filled circles represent intermediate hypothesized haplotypes between observed haplotypes, and lines between haplotypes represent a one-step mutational change. Dotted boxes represent one-step clades, dashed boxes represent two-step clades, solid line boxes represent three-step clades, and a thick black line separates the two four-step clades. Each clade has a hyphenated number, with the first number indicating the number of steps in the clade.

Restriction in mitochondrial gene flow is most likely due to isolation by geographic distance and would presumably be related to the short dispersal distances reported for *P. strobi* (Tomlin et al. 1997). When mean genetic distances are plotted against geographic distances between populations, these populations show a relationship between genetic and geographic distances on a regional scale in British Columbia (Fig. 3). However, this relationship does not hold over a Canada-wide scale, and many populations with a large geographic separation were not very diverged. For example, the Sandilands, Manitoba (MB), and Delaney, Nova Scotia (NS), populations are 2,550 km apart, whereas the Sechelt, BC, and Vernon, BC, samples are 340 km apart, and yet each pair had a percent sequence divergence of 0.69. In contrast to the pattern of differences between populations, variation in BC at the intrapopulation level is generally lower than in the rest of Canada (Table 5), which is consistent with

Table 2. Nested contingency analysis of geographical associations for COI sequence data from *P. strobi*

Clade	Permutational χ^2 statistic	Probability
1-2	7.341	0.123
1-3	0.600	1.000
1-7	5.000	0.114
1-9	3.343	0.293
1-14	6.000	0.079
1-15	8.000	0.029*
1-17	8.000	0.175
2-1	6.555	0.066
2-2	9.000	0.008*
2-3	12.000	0.002*
2-4	54.000	0.000*
2-5	13.000	0.000*
2-6	7.219	0.026*
2-7	7.000	0.036*
2-8	15.600	0.004*
2-9	18.703	0.001*
3-1	5.561	0.119
3-2	44.698	0.000*
3-3	13.765	0.015*
3-4	22.039	0.000*
4-1	49.397	0.000*
4-2	43.852	0.000*
Total	87.083	0.000*

Clades without geographic variation are not shown.
* Significant at the 0.05 level.

Table 3. Demographic inferences from nested clade distance analysis (Templeton et al. 1995; Templeton, 1998) of mtDNA in *P. strobi*

Clade	Inference chain	Inferred pattern
Haplotypes in 1-3	1-2-3-4-NO	A
Haplotypes in 1-9	1-2-3-4-NO	A
Haplotypes in 1-14	1-2-11-12-13-Yes	B
Haplotypes in 1-15	1-2-3-4-NO	A
One-step clades in 2-2	1-2-11-12-NO	C
One-step clades in 2-6	1-2-11-12-NO	C
One-step clades in 2-9	1-2-3-4-NO	A
Two-step clades in 3-4	1-2-11-12-YES	C
Three-step clades in 4-2	1-2-3-4-NO	A

A, restricted gene flow with isolation by distance; B, long-distance colonization; and C, contiguous range expansion.

Table 4. AMOVA results for tests of genetic divisions between populations of *P. strobi* in Canada

Source of variation	East vs. central vs. west		East/central vs. west		West	
	Variance components	% of variation	Variance components	% of variation	Variance components	% of variation
Among regions	0.752*	20.82	0.636*	16.74		
Among populations within regions	1.088**	30.14	1.386**	36.55	0.992*	41.39
Within populations	1.771**	49.04	1.771**	46.68	1.524**	58.61
Overall F_{ST}	0.50*		0.53*		0.41*	

* Significant at 0.05 level.
** Significant at 0.01 level.

prior suggestions that the range of *P. strobi* has expanded from east to west (Lewis et al. 2000). Thus, geographically separated populations seem to have had ample opportunity to diverge, resulting in new haplotypes, but the extent of these divergences may be obscured on a wider scale by historical events such as range expansion.

In fact, the second most common pattern inferred by nested clade analysis was contiguous range expansion. For clade 2-2, all haplotypes were from populations that occurred in the west, and in clade 2-6 and 3-2, the populations occurred within the eastern or central regions. These may be indicative of historical range expansions rather than current processes because the differences between eastern and western populations could only accumulate over a long time due to relatively slow rates of change in mtDNA (DeSalle et al. 1987).

The third pattern inferred from nested clade analysis was long-distance colonization. This seems to have been a low-frequency event, which supports prior findings that *P. strobi* does not normally disperse long distances (Tomlin et al. 1997). Humans could have mediated long-distance travel, or the pattern inferred may be a result of incomplete sampling in areas between eastern and western regions.

In our study, we found the same patterns of population structure by using mtDNA sequences that Lewis et al. (2000, 2001) described based on RAPD and isozyme markers. Western populations were significantly different from each other and were different from eastern populations, although the level of differentiation between populations was much greater in our study. Lewis et al. (2000) reported an F_{ST} value of 0.084, based on allozyme data, and our study showed an overall F_{ST} value of 0.50. Using the formula $Nm = (1\text{-}F_{ST})/2$ to determine the number of migrants per generation (Hartl and Clark 1989), the value from our study was 0.284, whereas 0.452 was the value in Lewis et al. (2001). Because our estimates of gene flow are for a maternal marker, the lower value from our study implies that females do not disperse as much as males. Thus, host differentiation may be increased because females tend not to disperse away from areas where they were reared. In any case, our study shows that mtDNA is an excellent source of information for population genetics studies, even if it is only one set of linked markers instead of many unlinked markers as in isozyme or RAPD studies.

Pest Management. Interpopulation mtDNA differences detected between *P. strobi* populations across Canada may reflect different selective pressures acting on relatively isolated populations (Namkoong et al. 1979), which may be due to the use of different hosts over its range. Differing selective pressures from hosts may be partially responsible for current genetic structuring of populations because individuals suited to hosts in a certain area would be selected for, but it is unlikely that individuals would be as suited for other host lines. This suggests the potential for variable population responses to control measures, a factor that

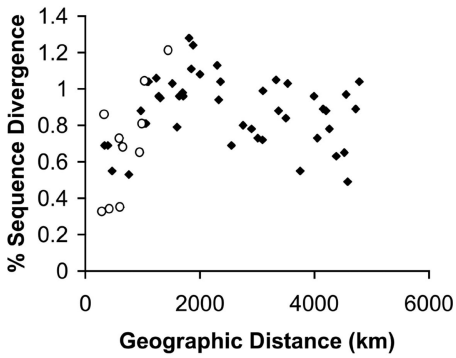

Fig. 3. Average percent sequence divergence between populations of *P. strobi* plotted against geographic distance. Hollow circles represent only BC population pairs (r^2 = 0.4183); filled diamonds represent other pairwise population combinations (all populations r^2 = 0.0147).

Table 5. Intrapopulation mean percent sequence divergences for COI of *P. strobi*

No.	Locality	% Sequence divergence
1	Kitimat, BC	0.197
2	Benson River, BC	0.322
3	Sechelt, BC	0.288
4	Fort Nelson, BC	0.322
5	Vernon, BC	0.465
6	Bertwell, SK	0.147
7	Sandilands, MB	0.522
8	Massey, ON	0.648
9	Launay, QC	0.682
10	Victoria County, NS	0.224
11	Delaney, NS	0.121

ON, Ontario; QC, Quebec; SK, Saskatchewan.

should be considered in the development of management tactics. The existence of geographic variability in resistance of lines of Sitka spruce to *P. strobi* in British Columbia (Tomlin and Borden 1996) may be correlated with genetic differences among weevil populations, which also can be investigated by examining the relative resistance of clonal lines of trees to different *P. strobi* haplotypes.

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