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AMPLIFIED MITOCHONDRIAL DNA AS A DIAGNOSTIC MARKER FOR SPECIES OF CONIFER-FEEDING *CHORISTONEURA* (LEPIDOPTERA: TORTRICIDAE)

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Abstract

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We describe a method for identifying conifer-feeding species and lineages of *Choristoneura* Lederer in Canada and Alaska. The method relies on amplification of mitochondrial (mt) DNA by the polymerase chain reaction (PCR); amplified DNA is then digested with restriction enzymes to give characteristic DNA fragment patterns. We used the cytochrome oxidase I and II genes of mtDNA, which were previously shown to contain numerous nucleotide differences at the level of species. Ten restriction enzymes were surveyed and a combination of two of these (*Eco*R V + *Hin*f I) was sufficient to distinguish the major mtDNA lineages. *Choristoneura fumiferana* (Clemens), *C. pinus* Freeman, and *C. rosaceana* (Harris) were readily distinguished from each other and from an assemblage of three putative western species (*C. occidentalis* Freeman, *C. orae* Freeman, and *C. biennis* Freeman). The three western species have the same mtDNA marker pattern in most individuals and, although ecologically differentiated, their populations may actually be conspecific. At one locality in Alaska, pheromone traps baited with lures for *C. fumiferana* attract moths with *C. fumiferana* mtDNA, and lures for *C. orae* attract moths with mtDNA that is characteristic of the western assemblage. This demonstrates geographic overlap of genetically distinct species in Alaska. The same two separate mtDNA lineages co-occur at two localities in Alberta, but pheromone attraction is unknown. In British Columbia, populations identified as *C. biennis* and *C. occidentalis* contain a few individuals with divergent mtDNA genotypes, the significance of which remains unclear. Amplified mtDNA thus provides a convenient, reliable marker for surveying genetic variation within species and for studying interactions among species of the *C. fumiferana* group.

Sperling, F.A.H., et D.A. Hickey. 1995. L'ADN mitochondrial amplifié, un outil d'identification des espèces de *Choristoneura* (Lepidoptera: Tortricidae) parasites des conifères. *The Canadian Entomologist* **127**: 277–288.

Résumé

Nous décrivons ici une méthode d'identification des espèces et des lignées de *Choristoneura* Lederer parasites des conifères au Canada et en Alaska. La méthode est basée sur l'amplification en chaîne des segments d'ADN mitochondrial (mt) par la polymérase; l'ADN amplifié est ensuite digéré par des enzymes de restriction, ce qui permet de reconnaître les fragments caractéristiques d'ADN. Nous avons utilisé les gènes cytochrome oxydase I et II de l'ADNmt, dans lesquels de nombreux nucléotides diffèrent selon l'espèce. Dix enzymes de restriction ont été utilisés et la combinaison de deux d'entre eux (*Eco*R V + *Hin*f I) s'est avérée suffisante pour distinguer la plupart des lignées d'ADNmt. *Choristoneura fumiferana* (Clemens), *C. pinus* Freeman et *C. rosaceana* (Harris) se distinguent facilement l'une de l'autre et se distinguent aussi d'un ensemble de trois espèces probables de l'ouest (*C. occidentalis* Freeman, *C. orae*

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Freeman et *C. biennis* Freeman). Les individus des trois espèces de l'ouest possèdent les mêmes fragments d'ADNmt et il est possible que les diverses populations, bien qu'écologiquement différentes, soient conspécifiques. À un endroit en Alaska, des pièges à phéromone ont été garnis de substances propres à attirer des *C. fumiferana* et ces pièges ont attiré des papillons à ADNmt de *C. fumiferana*; les pièges garnis de substances propres à attirer des *C. orae* ont attiré des papillons à ADNmt caractéristique du groupe des espèces de l'ouest. Il y a donc chevauchement géographique d'espèces génétiquement distinctes en Alaska. Les deux mêmes lignées d'ADNmt se retrouvent en deux localités d'Alberta, mais l'effet des phéromones à ces endroits est inconnu. En Colombie-Britannique, les populations identifiées comme *C. biennis* et *C. occidentalis* contiennent quelques individus à génotypes d'ADNmt différents, un phénomène qui reste inexpliqué. L'ADNmt amplifié est donc un marqueur commode et fiable dans les études de la variation génétique chez les diverses espèces et des interactions entre espèces au sein du groupe *C. fumiferana*.

[Traduit par la Rédaction]

Introduction

The eight species that comprise the spruce budworm species group [*Choristoneura fumiferana* (Clemens) group] include the most important conifer forest pests in Canada (Harvey 1985; Volney 1989; Moody 1992). Not surprisingly, members of the *C. fumiferana* group have been the subject of intense research effort over many years (Sanders et al. 1985). However, their phylogenetic relationships, species limits, and population structure remain poorly understood (Powell 1980; Harvey 1985; Dang 1992).

Previously, we surveyed sequence variation in mitochondrial DNA (mtDNA) of five species of the *C. fumiferana* group (Sperling and Hickey 1994). The five species were *C. fumiferana*, *C. pinus* Freeman, *C. occidentalis* Freeman, *C. biennis* Freeman, and *C. orae* Freeman (Fig. 1). That survey relied on 10 individuals sequenced over a 1.6-kilobase region and an additional 37 individuals sequenced over 470 base pairs. The most divergent mtDNA within the species group was found in *C. fumiferana*, while most individuals of three western species (*C. occidentalis*, *C. biennis*, and *C. orae*) showed little or no difference from each other. *Choristoneura pinus* was distinct, though related to the three western species. A small number of individuals from *C. occidentalis* and *C. biennis* populations had unusual,

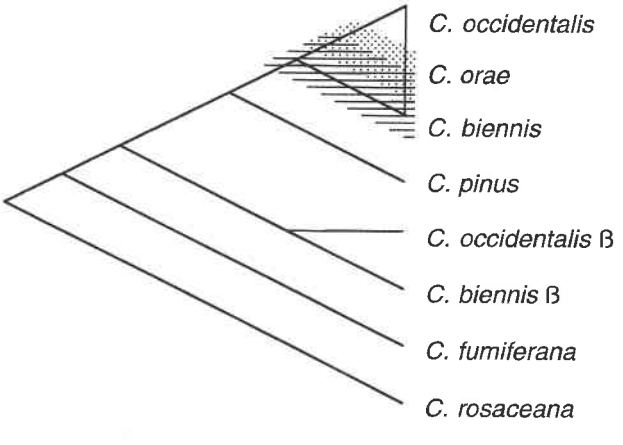


FIG. 1. Simplified cladogram of mtDNA relationships of conifer-feeding *Choristoneura*, based on mtDNA sequence (Sperling and Hickey 1994).

divergent mtDNA sequences (ß haplotypes). *Choristoneura rosaceana* (Harris) was used as an outgroup.

As an application of the sequence survey, we describe below a method that uses mtDNA to identify species and lineages of conifer-feeding *Choristoneura* in Canada and Alaska. Our purposes are (1) to provide an economical, robust technique for the identification of both adults and immatures and (2) to allow more extensive surveys of species limits and mtDNA lineage distributions than are possible using direct sequencing.

Materials and Methods

Collections. Populations of the *C. fumiferana* group were sampled for us by the Forest Insect and Disease Survey in Canada and the United States Forest Service in Alaska. Most specimens were collected as larvae and reared to the adult stage on artificial medium or on sprigs of their hostplant. Adults were frozen at –70°C until used. A small number of larvae were placed immediately in 95% ethanol. Samples from Alaska were collected by attracting adults to pheromone traps baited with 82:9:9 *E*11-14AC: Z11-14AC: *E*11-14OH, for *C. orae*, and 95:5 *E*:Z-11-14AL, for *C. fumiferana*. These pheromone-trapped adults were then placed in 95% ethanol, shipped, and kept at 4°C for up to 6 months before DNA extraction.

Twenty-two populations from five species of the *C. fumiferana* species group were sampled across Canada and Alaska (Table 1). One specimen of *C. rosaceana* was used from a 23rd locality. *Choristoneura rosaceana* is not part of the *C. fumiferana* group (Dang 1992) and was included to provide an outgroup. Its larvae feed occasionally on pines together with *C. pinus* (Otvos 1991).

DNA Methods. Total genomic DNA was extracted from individual adult thoraces by a modification of the technique of Harrison et al. (1987). Wings and abdomens were broken off and dried in gelatin capsules. These are deposited as voucher specimens at the Canadian National Collection, Agriculture Canada, Ottawa. Thoraces were ground into powder using disposable plastic pestles inside 1.5-mL microfuge tubes immersed in liquid nitrogen. Lifton

TABLE 1. Localities sampled, grouped by preliminary identifications based on larval hostplant or adult pheromone attractant

Preliminary identification	Larval hostplant or adult pheromones	Localities*
Choristoneura fumiferana	Balsam fir	Blanch River, Newfoundland (10); Kedgwick, New Brunswick (5); Holmes Lake, New Brunswick (5)
C. fumiferana	White spruce	Mt. Carleton, New Brunswick (5); Causapscal, Quebec (5); Ignace District, Ontario (10); Carberry, Manitoba (10); Cypress Hills, Alberta (16); Red Lodge, Alberta (13); Hawk Hills, Alberta (21)
C. fumiferana	95:5 *E*:Z-11-14AL	Fairbanks area, Alaska (10)
C. orae	82:9:9 *E*:ZAC:OH	Fairbanks area, Alaska (10)
C. biennis	Alpine fir	Morrisey Creek, BC (11); McBride, BC (10)
C. biennis	Englemann spruce†	Numa Falls, BC (10)
C. occidentalis	Douglas fir‡	Monte Creek, BC (11); Winfield, BC (11); Bridesville, BC (9); Greenwood, BC (10); Clearbrook, BC (11)
C. pinus	Jack pine	Parry Sound, Ontario #1 (10) and #2 (10)
C. rosaceana§	At light	Ste. Agathe, Quebec (1)

*Numbers in parentheses are sample size; BC = British Columbia.
†Some individuals from Numa Falls were also collected on Alpine fir.
‡Some individuals from Bridesville were also collected on Lodgepole pine.
§*Choristoneura rosaceana* was identified by adult wing pattern.

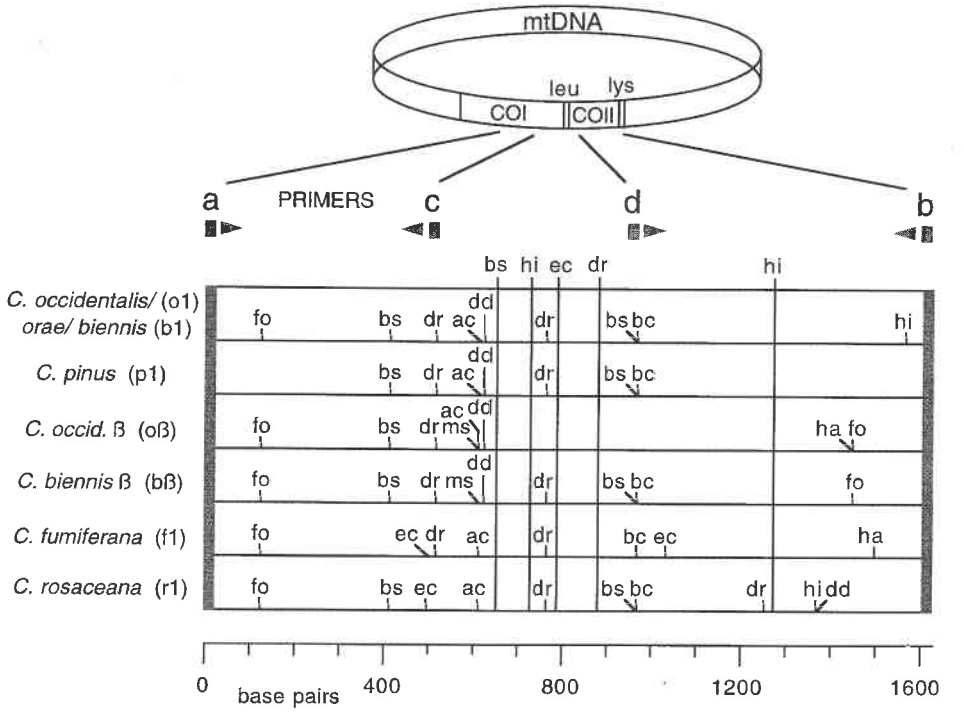


FIG. 2. Schematic showing region of mtDNA amplified, location of primers, and locations of diagnostic restriction enzyme sites predicted from sequences of major mtDNA lineages. Enzyme symbols consist of the first two letters of each enzyme name. Primer sequences are as follows, with primer locations (in parentheses) as in Simon et al. (1994):

a. (C1-J-2183) 5′ CAACATTTATTTTGATTTTTTGG 3′
b. (C1-N-2659) 5′ GAGACCATTACTTGCTTTCAGTCATCT 3′
c. (C2-J-3138) 5′ GCTAATCCAGTGAATAATGG 3′
d. (TK-N-3782) 5′ AGAGCCTCTCCTTTAATAGAACA 3′

buffer (800 µL of 0.1 *M* tris buffer, 0.2 *M* sucrose, 0.05 *M* ethylaminediaminetetraacetate (EDTA), 0.5% sodium dodecyl sulfate, pH 9.0) was added to the powder, and the homogenate was lightly mixed by vortex and incubated at room temperature for 15 min or more. Then 120 µL of 8 *M* potassium acetate was added, and the tubes were inverted and placed on ice for 10 min or more. The resulting precipitate was centrifuged for 15 min and the supernatant was decanted to a fresh tube. The supernatant fluid was extracted with one volume of phenol, followed by extraction of the aqueous layer with one volume of 24:1 chloroform : isoamyl alcohol. DNA was precipitated by addition of one volume of isopropanol followed by 25 min of centrifugation. The pellet was washed in 70% ethanol and resuspended in 200 µL TE buffer (10 m*M* TrisHCl buffer, 1 m*M* EDTA, pH 8.0). Samples preserved in ethanol were processed as above, except thoraces of adults or head capsules of larvae were vacuum-dried to remove ethanol before grinding in liquid nitrogen. The final DNA pellet from ethanol-preserved specimens was resuspended in only 100 µL of TE buffer.

DNA regions and primers used for amplification are shown in Figure 2. Specific DNA fragments were amplified using the polymerase chain reaction (PCR) as per instructions contained in Perkin Elmer Cetus GeneAmp© Reagent Kit. One microlitre of DNA in TE was used as template, in a total reaction volume of 50 µL overlain by one drop of light mineral oil. Taq polymerase was added after an initial incubation at 95°C for 3 min in a Perkin-Elmer Cetus thermal cycler, during an annealing phase of 45°C for 2 min, and before

an extension phase of 72°C for 1.5 min. This was followed by 30–35 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1.5 min, and terminated with one cycle that was the same as the previous ones except for an extension of 5 min.

Yield and length of DNA from the PCR reaction were determined by comparison to a 123-bp ladder (Gibco BRL) in a 1.0% agarose gel. For small fragments of up to 600 bp, no consistent difference in PCR yield was found for template DNA from alcohol or frozen specimens. However, the full-length fragment of 1.6 kb was consistently produced only for DNA from frozen specimens. In the rare cases in which no product was obtained for DNA from frozen specimens, a second PCR reaction was usually successful once the template DNA had been re-extracted with phenol/chloroform and reprecipitated with alcohol. No product was usually formed if more than 2 μL of DNA in TE was used as template, perhaps because the EDTA in TE interfered with the magnesium cofactor in the PCR buffer.

About 1 μg of the PCR fragment that was obtained (usually 1–4 μL of the DNA suspension) was digested using restriction endonucleases in a total volume of 15 μL as per recommendations of suppliers (generally New England Biolabs). For example, 2 μL of the PCR product was added to 11.2 μL of water, 1.5 μL of reaction buffer (supplied), and 0.3 μL of enzyme in a 0.5-mL microfuge tube, then incubated at 37°C for 2 h. DNA fragments were then separated by size by electrophoresis in 1.0–1.5% agarose gels and visualized by staining with ethidium bromide (Sambrook et al. 1989). In many cases, faint bands were produced that were not part of the normal, strongly visible fragment pattern. These spurious bands were due to either small amounts of non-specific product from the original PCR reaction or later partial digestion by restriction enzymes. Expected bands were recognized by direct calculation of length from the published sequences (Sperling and Hickey 1994) and by verifying that the sizes of observed bands summed to the size of the undigested fragment. As with DNA quantification for the PCR reaction, sizes of digested fragments were estimated by comparison with the 123-bp ladder.

Survey Strategy. Our restriction site survey focussed on a 1.6-kb region of mtDNA that had been sequenced previously in one or two individuals of each species [Sperling and Hickey (1994): sequences deposited as GenBank Nos. L19094-9]. This mtDNA region encompassed half of the cytochrome oxidase I gene and all of the cytochrome oxidase II gene. Using the Microgenie sequence analysis program (Beckman Instruments, Inc.), we found 10 restriction enzymes that produced distinct restriction fragment patterns for different species (Fig. 2). Two enzymes were assayed by digesting DNA fragments with only a single enzyme at a time (*Bsp*H I and *Dra* I). The remaining eight enzymes were generally assayed in four pairs (1, *Eco*R V + *Hin*f I; 2, *Bcl* I + *Dde* I; 3, *Hae* III + *Msp* I; and 4, *Acc* I + *Fok* I).

Restriction sites were surveyed in 5–21 individuals of 22 populations of the *C. fumiferana* species group (Table 1). A total of 204 specimens was characterized using mtDNA restriction sites across the 1.6-kb region. The DNA of an additional 20 specimens (from Alaska) was partially degraded and could not be PCR-amplified across the full 1.6-kb region. However, a region of 0.6 kb was successfully amplified from these specimens and characterized using four diagnostic restriction enzymes (*Eco*R V, *Fok* I, *Hin*f I, and *Hae* III). DNA from larvae preserved in ethanol was also reliably amplified only for smaller fragments (primers a+c and d+b).

Some specimens produced fragment patterns that were not predicted from the previous survey of sequence variation. The exact location of each new restriction site was determined by sequencing the appropriate region. For new restriction fragment patterns, one specimen displaying this pattern was also sequenced over the region of 470 bp enclosed by primers a+c. That gave a total of 56 individuals that were directly sequenced over 470 bp as well as characterized by restriction sites over the entire 1.6-kb region. The combination of sequence and restriction site information for these 56 individuals gave 27 different mtDNA haplotypes (= mtDNA genotypes).

Divergences within species or lineages were estimated with the iterative estimation technique of Nei and Tajima (1983). Nucleotide and haplotype diversity were estimated with formulae 8.4, 8.12, 10.5, and 10.9 of Nei (1987). Divergences between species were not calculated because of the bias caused by surveying only sites that potentially distinguished species. Phylogenetic relationships were analyzed using PAUP (Swofford 1993), using all default parameters. Restriction sites from the unsequenced region and nucleotide positions from the first 470 bp were combined and treated as unordered characters with six possible states (0, 1, A, T, C, and G).

Results

Restriction Site Survey. Restriction site maps for the 10 enzymes used to survey variation are shown in Figure 3. Fragment lengths include the primers at each end of the original amplified fragment. Most of the single-enzyme fragment patterns coincided with those predicted from sequence information (Fig. 2). A small number of single-enzyme fragment patterns were new (Ec.b, Hf.c, and Fo.b), and numerous new haplotypes were generated by combining all 10 patterns.

The geographic distribution of restriction site haplotypes showed a pattern in which a single haplotype was common and widely distributed within each of *C. fumiferana* (fa), *C. pinus* (pa), and the western assemblage (oa) (Fig. 4). Except for the western edge of the range of *C. fumiferana*, all variants except the fc, od, and bß haplotypes were found only once within each species. Two individuals were found for each of the fc and od haplotypes, and in both cases these haplotypes occurred in different populations. Three individuals with the bß haplotype were found, including two in one population and one in another.

Three locations produced a mixture of haplotypes. The Cypress Hills in southern Alberta and Red Lodge Park in west-central Alberta had populations with both fa and oa haplotypes, and the Fairbanks area in Alaska had fa* and oa* haplotypes. The pheromone lures for *C. fumiferana* and *C. orae* that were used to trap moths in Alaska attracted males with mtDNA haplotypes characteristic of *C. fumiferana* and the western assemblage, respectively, indicating that two species occur there in sympatry. Information on pheromone attractiveness was not obtained for the Alberta populations.

One population sample that was preliminarily identified as *C. pinus* on the basis of larval hostplant contained a single individual with a haplotype indistinguishable from *C. rosaceana*. Examination of the wings kept as vouchers for this specimen revealed that it was indeed *C. rosaceana*, thereby demonstrating the value of mtDNA as an independent marker for sorting individuals from mixed collections.

Based on restriction site haplotypes, all species and populations showed low genetic diversities (Table 2). Haplotype diversity and nucleotide diversity estimates were higher for *C. occidentalis* and *C. biennis* (when ß haplotypes were included) than for *C. fumiferana*

TABLE 2. Genetic diversity within species or major mtDNA lineages*

	Sample size	Haplotype diversity ($h \pm 2$ SE)	Nucleotide diversity ($\pi \pm 2$ SE)
fumiferana (fa-fe)	97	0.100±0.238	0.0008±0.0092
All *occidentalis*	54	0.242±0.237	0.0040±0.0228
occidentalis w/o oß	53	0.211±0.255	0.0031±0.0031
All *biennis*	31	0.235±0.316	0.0056±0.0050
biennis w/o bß	28	0.069±0.467	0.0004±0.0028
pinus (pa-pb)	19	0.102±0.532	0.0004±0.0033

*Calculated with equations 8.4, 8.12, 10.5, and 10.9 of Nei (1987).

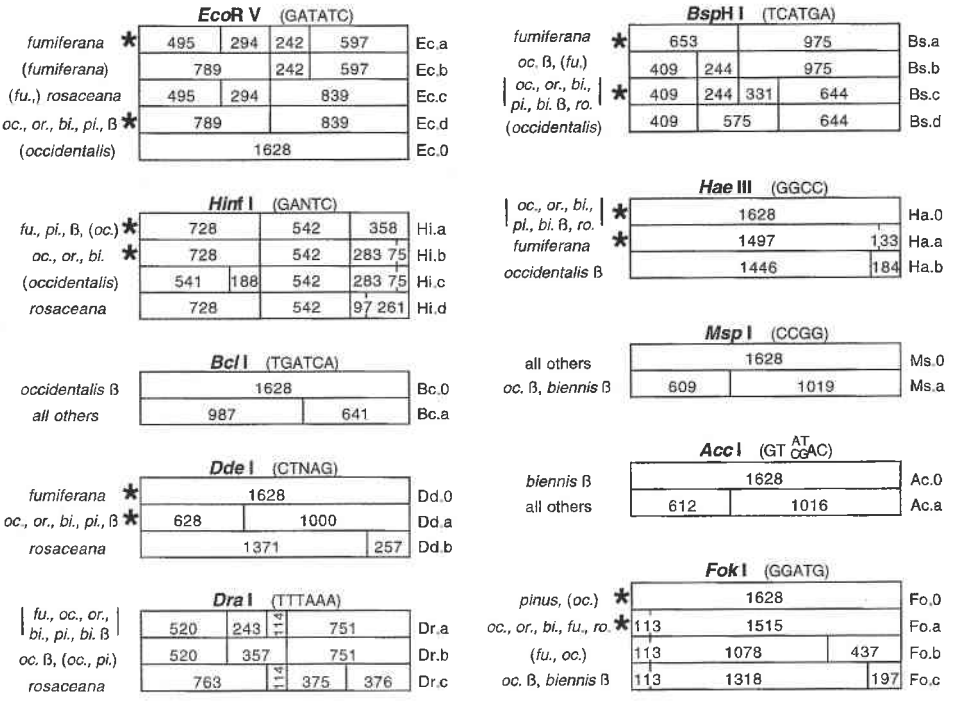


FIG. 3. Restriction site locations and fragment sizes for 10 enzymes. Species showing each fragment pattern are to the left of each block, with rare minor variants enclosed in parentheses. Abbreviations: *fu.* = *fumiferana*, *oc.* = *occidentalis*, *or.* = *orae*, *bi.* = *biennis*, ß = both *occidentalis* ß and *biennis* ß, *ro.* = *rosaceana*. Bold stars indicate common patterns that are useful for species separations within the *C. fumiferana* group. Letters to the right of each block indicate single-enzyme fragment patterns. Haplotypes defined by combinations of fragment patterns are as follows, with minor variants in parentheses and differences from major lineage patterns underlined:

fa = Ec.a + Hi. a + Bc.a + Dd.0 + Dr.a + Bs.a + Ha.a + Ms.0 + Ac.a + Fo.a
(fb = Ec.b + Hi. a + Bc.a + Dd.0 + Dr.a + Bs.a + Ha.a + Ms.0 + Ac.a + Fo.a)
(fc = Ec.a + Hi. a + Bc.a + Dd.0 + Dr.a + Bs.a + Ha.a + Ms.0 + Ac.a + Fo.b)
(fd = Ec.c + Hi. a + Bc.a + Dd.0 + Dr.a + Bs.a + Ha.a + Ms.0 + Ac.a + Fo.a)
(fe = Ec.a + Hi. a + Bc.a + Dd.0 + Dr.a + Bs.b + Ha.a + Ms.0 + Ac.a + Fo.a)
oß = Ec.d + Hi. a + Bc.0 + Dd.a + Dr.b + Bs.b + Ha.b + Ms.a + Ac.a + Fo.c.
bß = Ec.d + Hi. a + Bc.a + Dd.a + Dr.a + Bs.c + Ha.0 + Ms.a + Ac.0 + Fo.c.
pa = Ec.d + Hi.a + Bc.a + Dd.a + Dr.a + Bs.c + Ha.0 + Ms.0 + Ac.a + Fo.0.
(pb = Ec.d + Hi.a + Bc.a + Dd.a + Dr.b + Bs.c + Ha.0 + Ms.0 + Ac.a + Fo.0)
oa = Ec.d + Hi.b + Bc.a + Dd.a + Dr.a + Bs.c + Ha.0 + Ms.0 + Ac.a + Fo.a.
(ob = Ec.d + Hi.b + Bc.a + Dd.a + Dr.a + Bs.c + Ha.0 + Ms.0 + Ac.a + Fo.0)
(oc = Ec.d + Hi.a + Bc.a + Dd.a + Dr.a + Bs.c + Ha.0 + Ms.0 + Ac.a + Fo.b)
(od = Ec.0 + Hi.b + Bc.a + Dd.a + Dr.a + Bs.c + Ha.0 + Ms.0 + Ac.a + Fo.a)
(oe = Ec.d + Hi.b + Bc.a + Dd.a + Dr.b + Bs.c + Ha.0 + Ms.0 + Ac.a + Fo.a)
(of = Ec.d + Hi.b + Bc.a + Dd.a + Dr.a + Bs.d + Ha.0 + Ms.0 + Ac.a + Fo.a)
(og = Ec.d + Hi.a + Bc.a + Dd.a + Dr.a + Bs.c + Ha.0 + Ms.0 + Ac.a + Fo.a)
ra = Ec.c + Hi.d + Bc.a + Dd.b + Dr.c + Bs.c + Ha.0 + Ms.0 + Ac.a + Fo.a

and *C. pinus*, but differences among species were not statististically significant. In fact, because of small populations and low genetic diversities, only the estimates of haplotype diversity for *C. occidentalis* and nucleotide diversity for *C. biennis* were significantly different from zero.

No single restriction enzyme distinguished all the major species and lineages. However, one pair of restriction enzymes (*Eco*R V and *Hin*f I) provided a convenient separation of

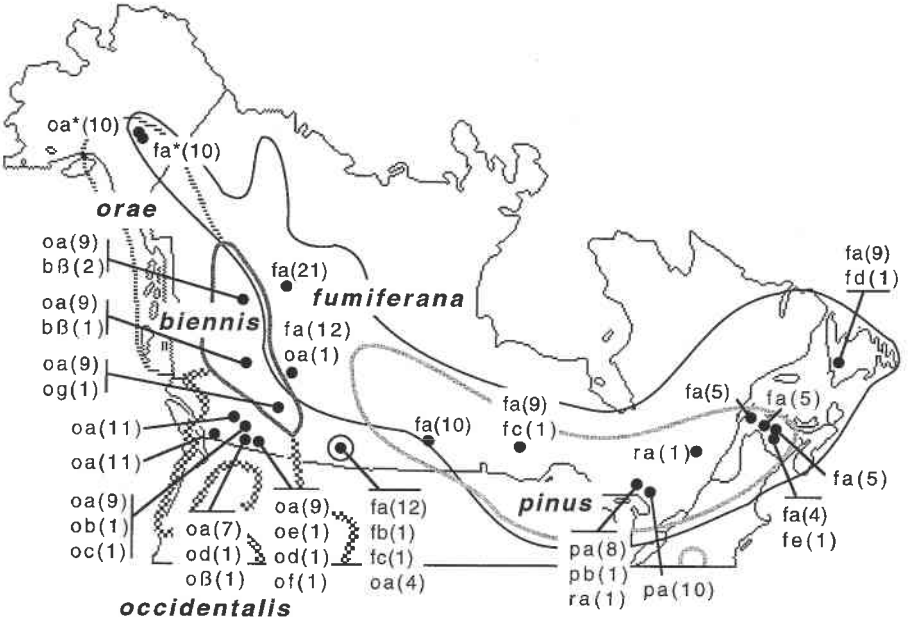
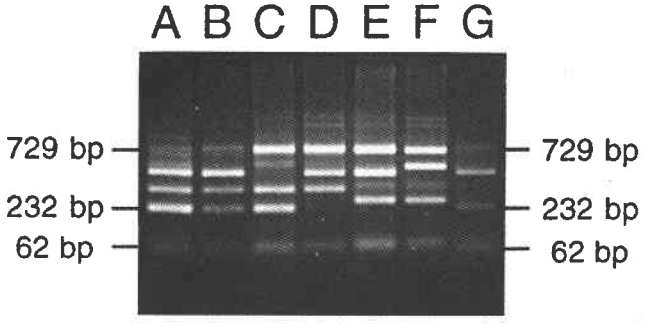


FIG. 4. Geographic distribution of mtDNA restriction site haplotypes (defined in Fig. 3). Outlines of *Choristoneura* species ranges are based on Harvey (1985). Haplotypes designated fa* and oa* are identical to fa and oa except they were assayed using *Eco*R V, *Fok* I, *Hae* III, and *Hin*f I on a fragment produced using primers d+b.



b.

		*Eco*R V	*Hin*f I	*Eco*R V	*Eco*R V	*Hin*f I	
A	(Ec.a + Hi.a)	497 | 232	62	242 | 238		357	*fumiferana*
B	(Ec.c + Hi.a)	497 | 232	62	480		357	*(fumiferana)*
C	(Ec.b + Hi.a)	729	62	242 | 238		357	*(fumiferana)*
D	(Ec.d + Hi.a)	729	62	480		357	*pinus* & ß's
E	(Ec.d + Hi.b)	729	62	480		283 | 74	*occidentalis/ orae/ biennis*
F	(Ec.0 + Hi.b)	729		542		283 | 74	*(occidentalis)*
G	(Ec.c + Hi.d)	497 | 232	62	480		97 | 260	*rosaceana*

FIG. 5. (*a*) (Top) Gel photo of fragments produced by digestion of 1.6-kb PCR product using *Eco*R V and *Hin*f I. DNA fragments are separated by size in a 2% agarose gel. Letters at the top of each lane refer to the patterns mapped in Fig. 5*b*. (*b*) Restriction site maps for the seven *Eco*R V + *Hin*f I patterns photographed. Numbers show length of fragments in base pairs.

species as well as some information on variation within species. Fragment patterns remained interpretable when amplified DNA was digested with both enzymes simultaneously (Fig. 5). Only *C. pinus* mtDNA and the ß haplotypes were not differentiated using these two enzymes. However, *C. pinus* and the ß haplotypes were found in geographically separate regions, and any uncertain cases can easily be assayed using other enzymes.

DNA from adult and larval specimens that were preserved in ethanol remained assayable for at least 2 years. Although there was some degradation and many of these samples could not be amplified over the full 1.6-kb region, specimens were readily identified by amplifying fragments of less than 600 bp. For such samples, primers a+c amplified a fragment that was most useful for sequence analysis, while primers d+b amplified a fragment that was especially informative in restriction site analyses.

Phylogenetic Relationships. Restriction sites were combined with DNA sequences to give information on phylogenetic relationships. This was because sites were chosen primarily to provide diagnostic characters for single major lineages and relatively few shared derived site characters were surveyed. Two new sequence haplotypes were found over the 470-bp region sequenced in individuals with new restriction sites. These were designated as the o8 haplotype, which differed from the o1 haplotype by a mutation to G in position 315, and the o9 haplotype, which differed from the b2 haplotype by a T in position 57 [numbering here follows Sperling and Hickey (1994); fragment lengths indicated elsewhere include an additional 27 bp for the COI primer region].

Parsimony analysis gave two most-parsimonious trees for the 27 haplotypes distinguished by unique sequence and restriction site combinations. One of these trees is shown in Figure 6. The second tree had the of/09 and og/b2 haplotypes reversed in position. *Choristoneura fumiferana* haplotypes formed a distinct group characterized by the presence

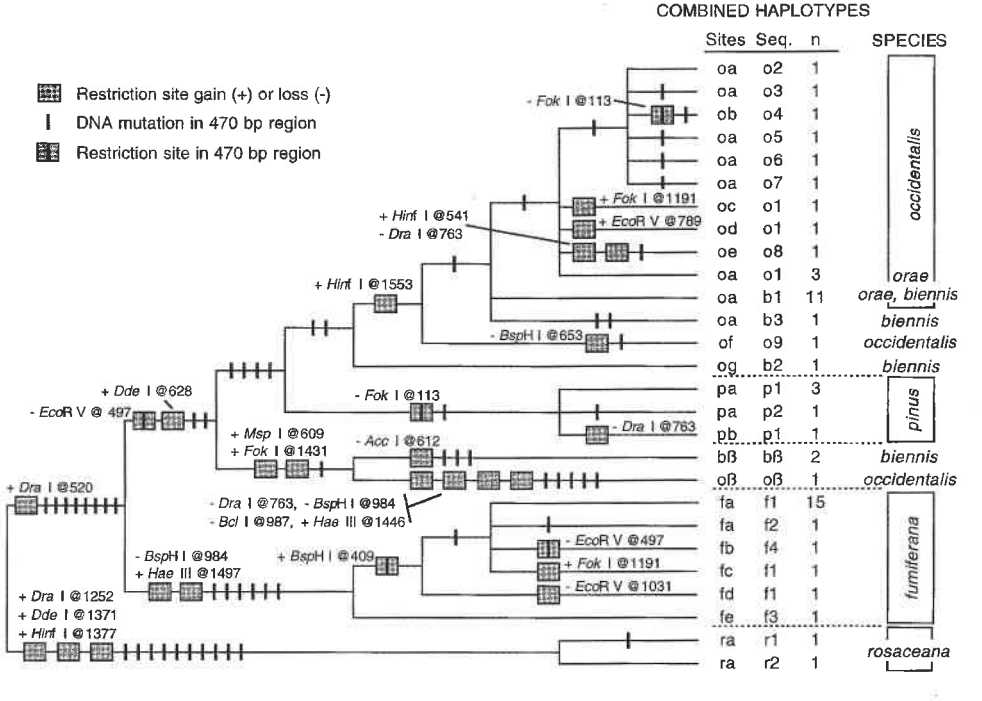


FIG. 6. Relationships of mtDNA of conifer-feeding *Choristoneura* in Canada and Alaska, based on combined sequence and restriction site data.

of one restriction site (*Hae* III @1497) and the loss of another (*Bsp*H I @984). Neither of these two sites showed any homoplasy. The three haplotypes found in *C. pinus* were characterized by a single site loss (*Fok* I @113) and this site appears to have been independently lost in one individual of *C. occidentalis* (ob/04). Except for the single individual with the og/b2 haplotype, the western assemblage comprising *C. orae* and most of *C. biennis* and *C. occidentalis* was characterized by the presence of a site that showed no homoplasy (*Hinf* I @1553). The two ß haplotypes were characterized by the presence of two restriction sites (*Msp* I @609 and *Fok* I @1431).

The addition of restriction site information thus confirmed the phylogeny hypothesized using a smaller number of individuals and sequence alone (Sperling and Hickey 1994). The mtDNA of *C. fumiferana* was the first to branch off, followed by the ß haplotypes of *C. biennis* and *C. occidentalis* and then the mtDNA of *C. pinus*. The separation between *C. occidentalis* and *C. biennis* was less obvious than before. In fact, a new population of *C. occidentalis* collected on Douglas fir at Clearbrook, B.C., contained ao/b1 haplotypes, which had previously been found only in *C. biennis* and *C. orae*.

Discussion

Mitochondrial DNA has proven useful for elucidating genetic variation and species limits in a variety of insect species complexes (e.g. Martin and Simon 1990; Hall and Smith 1991; Bogdanowisz et al. 1993; Sperling 1993; Vogler et al. 1993; Boyce et al. 1994; Sperling and Harrison 1994; Simon et al. 1994). Its maternal inheritance, abundance, and relative stability make it ideal as a diagnostic marker (Avise 1991; but see Hurst and Hoekstra 1994). However, the molecule may not always accumulate mutations rapidly enough to be informative at the level of populations. One way that the usefulness of mtDNA can be extended is to perform an intensive survey of sequence variation in a small number of individuals (Simon et al. 1993). Such sequence information allows the selection of potentially diagnostic restriction sites in small amplified fragments, as well as the construction of primers that are located to facilitate amplification of partially degraded material.

Mitochondrial DNA of the spruce budworm species group provides an illustration of the utility of this approach. *Choristoneura fumiferana*, *C. pinus*, and the western assemblage are separated from each other, and mtDNA restriction sites provide some information about variation within species. A few divergent mtDNAs were also found in *C. biennis* and *C. occidentalis*, and these may be variously interpreted as representing cryptic species, retained ancestral polymorphisms, rare introgression from unknown related species, or remnant haplotypes that have survived introgressive innundation of mtDNA from invading species (see Sperling and Hickey 1994 for discussion). In summary, of the five currently recognized species in the spruce budworm species complex, three groups of populations are readily distinguished using mtDNA.

The mtDNA assay is quite simple, inexpensive (1 to 2 dollars per specimen), and effective using a convenient preservation method. In fact, using ethanol-preserved material, amplified mtDNA provided the first genetic evidence for the co-occurrence of *C. fumiferana* with *C. orae* in Alaska. In contrast, most previous genetic investigations of *Choristoneura* species have been done on populations that were believed to be monospecific. Because morphological characters have not served to identify species consistently, and allozymes require special preservation, the extent to which species interact at the edge of their range has remained poorly understood. Using mtDNA, it is now feasible to survey larger numbers of specimens efficiently. In particular, it will be interesting to determine the nature and extent of interactions between *Choristoneura* species along the foothills of the Rocky Mountains in Alberta and northern British Columbia. The fact that low diversity in mtDNA was found within species or major lineages, even over the vast geographic range of *C. fumiferana*,

implies that mtDNA is likely to remain widely useful as a marker of species boundaries in species that have not yet been sampled throughout their range, such as *C. pinus*.

Like any single marker, however, mtDNA may not always mirror species boundaries (e.g. Sperling 1993). This may occur because of gene introgression between species, rapid speciation, or random sorting of retained ancestral polymorphism (Avise 1991). It remains important to view variation in mtDNA in the context of patterns in other characters. The western species of the *C. fumiferana* group are particularly enigmatic in this respect. Variation in mtDNA does not conform to currently used species concepts, but rather suggests that *C. occidentalis, C. biennis*, and *C. orae* are recently differentiated populations that have no significant barriers to gene flow other than isolation by distance. Earlier evidence from allozymes also indicates a very close or conspecific relationship among the western species (Stock and Castrovillo 1981; Harvey 1985) and new data from allozymes (G. Harvey personal communication) show no substantive disagreement with the mtDNA-based phylogeny. However, speciation may be potentially rapid in *Choristoneura*, because most genes that have been implicated in species differences are sex linked and therefore have the opportunity to form linkage complexes that are susceptible to rapid selection (Sperling 1994). If this is true, mtDNA may simply not be a sufficiently sensitive indicator of speciation in some of these species.

On the other hand, the western *Choristoneura* species contain a few individuals with unusual mtDNA (β haplotypes). Other genetic markers will be needed to determine whether these specimens represent normal variation within species, range extensions for southern species such as *C. lambertiana* (Busck), or new species (e.g. Gray and Slessor 1989; Gray and Gries 1993). Because one of the specimens (oβ haplotype) came from a population of *C. occidentalis* with unusual host associations on lodgepole pine, it seems reasonable to expect that variation in mtDNA will continue to provide a useful tool for understanding this notoriously complex group of species.

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