MITOCHONDRIAL DNA VARIATION AND IDENTIFICATION OF BARK WEEVILS IN THE *PISSODES STROBI* SPECIES GROUP IN WESTERN CANADA (COLEOPTERA: CURCULIONIDAE)

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Abstract

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Morphological, allozyme, and chromosomal characters and ecological traits have limited value for discriminating among four closely related *Pissodes* spp. known from western Canada. We amplified a 1585-bp segment of mitochondrial DNA (mtDNA), including half of the cytochrome oxidase I (COI) and all of the tRNA leucine and COII genes, using the polymerase chain reaction, and studied mtDNA variation within and among all four species of *Pissodes* with restriction enzymes. Twenty-four haplotypes were found among the 121 maternal lineages surveyed. Haplotype distributions suggest intermediate levels of gene flow for each species. Interspecific estimated sequence divergences ranged from 0 to 28.7%. Phylogenetic relationships among species were reconstructed using *P. affinis* Randall as an outgroup. *Pissodes terminalis* Hopping and *P. nemorensis* Germar were the most closely related species, and this clade was most closely related to *P. strobi* (Peck); *P. schwarzi* Hopkins branched off below these three. Restriction site variation is sufficient to discriminate unambiguously among most species. However, *P. terminalis* and *P. nemorensis* haplotypes were very similar, which may complicate discrimination between these two species, using mtDNA characters, where their ranges putatively overlap in Manitoba. A diagnostic protocol using three restriction enzymes, *Bcl* I, *Dra* I, and *Hinf* I, is recommended.

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Résumé

Les allozymes, les caractères morphologiques et chromosomiques ou encore les propriétés écologiques ne permettent pas de distinguer les quatre espèces très apparentées de *Pissodes* spp. de l'ouest du Canada. Nous avons amplifié un segment de 1585 paires de base d'ADN mitochondrial (ADNmt), incluant la moitié des gènes codant pour la cytochrome oxydase I (COI) de même que tous les gènes codant pour l'ARNt-leucine et pour la cytochrome oxydase II, au moyen de la réaction en chaîne par la polymérase, et avons par la suite étudié la variation de l'ADNmt au sein de chacune des espèces et entre les quatre espèces de *Pissodes* au moyen d'enzymes de restriction. Vingt-quatre haplotypes ont été trouvés parmi les 121 lignées maternelles étudiées. La répartition des haplotypes a permis de constater qu'il se fait un transfert de gènes d'intensité moyenne chez chacune des espèces. Les divergences interspécifiques entre les séquences ont été évaluées entre 0 et 28,7%. Les relations phylogénétiques entre les espèces ont été reconstituées en utilisant *P. affinis* Randall comme groupe externe. *Pissodes terminalis* Hopping et *P. nemorensis* Germar sont les deux espèces les plus apparentées et ce clade s'apparente surtout à *P. strobi* (Peck); *P. schwarzi* Hopkins est apparu avant les trois autres espèces. La variation des sites de restriction permet de faire une discrimination non équivoque entre la plupart des espèces. Cependant, les haplotypes de *P. terminalis* et de *P. nemorensis* sont très semblables, ce qui peut créer de la confusion entre les deux

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espèces lorsqu'elles sont identifiées par l'ADNmt aux endroits où elles semblent cohabiter au Manitoba. Un protocole diagnostique basé sur trois enzymes de restriction, *Bcl* I, *Dra* I et *Hinf* I est proposé.

[Traduit par la Rédaction]

Introduction

The genus *Pissodes* is distributed throughout North America, Europe, and Northern Asia (Hopkins 1911; Gemminger and Harold 1871; C.W. O'Brien 1989), and contains 55–60 species, some of which are undescribed (Langor, unpublished). All 21 species in Canada and the United States (L.F. O'Brien 1989) infest the bark and outer wood of coniferous trees, and several species are important forest pests (Hopkins 1911). The white pine weevil, *P. strobi*, and the lodgepole terminal weevil, *P. terminalis*, are important pests of *Pinus* and *Picea*, especially young trees (Belyea and Sullivan 1956; Langor et al. 1992). These two species infest and kill the terminal leaders, resulting in growth loss and crooking or branching of the trunk (Drouin et al. 1963; Alfaro and Ying 1990; Langor et al. 1992). The deodar weevil, *P. nemorensis*, also occasionally infests terminal leaders but is more commonly associated with lower portions of the stem and root collar (Fontaine et al. 1983; Atkinson et al. 1988). These three species and *P. schwarzi*, another bole-inhabiting species (Hopkins 1911), have been informally grouped into the *P. strobi* species group (Smith and Takenouchi 1969). They are very similar morphologically, and some species readily interbreed to form viable offspring in laboratory crosses (Godwin and Odell 1967; Smith and Takenouchi 1969; Phillips and Lanier 1983).

Hopkins (1911) recognized 11 species of *Pissodes* in subsection b2, the subsection containing three of the above-mentioned species (*P. terminalis* was not recognized by Hopkins). Many of Hopkins' species were described on the basis of host associations and geography. He used scale patterns as the principal morphological characters for discriminating among these species, but these are highly variable and unreliable as good diagnostic characters for species in the *P. strobi* species group (Langor, unpublished). Six of the taxa in subsection b2 were later synonymyzed (Smith and Sugden 1969; Phillips et al. 1987) and the status of two rare species, *P. californicus* Hopkins and *P. webbi* Hopkins, is uncertain. As part of a taxonomic revision of the genus *Pissodes*, morphological variation in the *P. strobi* species group is under study by the senior author.

Studies (summarized by Smith and Virkki 1978) show that chromosomes have diagnostic value; however, cytogenetic characters can be studied only in fresh insect material. The chromosome complement of *P. terminalis* is unique in that the species is sexually dimorphic for one of its autosomes; males (2N = 29) are heterozygous for the presence of a fusion of two acrocentric chromosomes and females (2N = 28) are homozygous for the fusion product (Smith and Takenouchi 1962). *Pissodes schwarzi* also has a chromosome complement of 2N = 28 but does not exhibit sexual dimorphism (Smith and Virkki 1978). The chromosome number of *P. strobi* is 2N = 34. The chromosome number for *P. nemorensis* is polymorphic at 2N = 30–34, with lower numbers resulting from centric fusion (Smith and Virkki 1978).

Analysis of allozyme variation indicates little differentiation in allozymes among populations and species of *P. strobi*, *P. terminalis*, and *P. nemorensis*., and no fixed alleles (Phillips 1984). Also, assessment of allozyme variation is limited to fresh or frozen insect material.

Ecological traits, such as host and habitat preference, are often useful for discriminating among species. *Pissodes nemorensis* and *P. schwarzi* usually infest the lower boles of trees, whereas *P. terminalis* and *P. strobi* infest the terminal leaders. At this time it appears that the ranges of the two bole-inhabiting species do not overlap (Fig. 1). The two terminal-infesting species can often be separated based on host associations. In western North America, where these two species overlap, *P. terminalis* is found exclusively in *Pinus*, whereas *P. strobi* is found mainly in *Picea*, but also occasionally in *Pinus*. These two species can also be

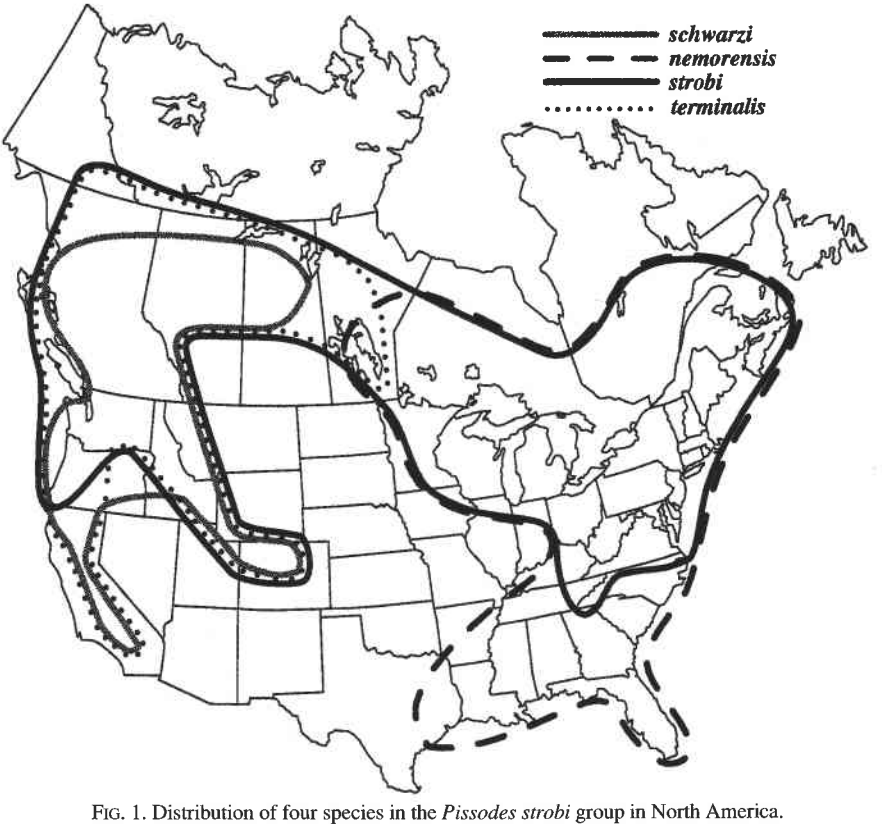

FIG. 1. Distribution of four species in the *Pissodes strobi* group in North America.

separated based on behavioral characters. *Pissodes terminalis* attacks the current year's terminal, lays eggs in the lower half of the terminal, and larvae generally feed upward and solitarily (Langor et al. 1992). *Pissodes strobi* attacks the previous year's terminal, lays eggs in the upper half of the terminal, and larvae generally feed downward and gregariously (Belyea and Sullivan 1956); however, specimens submitted for identification rarely have ecological or biological data associated with them.

In recent years, mitochondrial DNA (mtDNA), by virtue of its simple structure, maternal inheritance, and relatively rapid evolutionary rates, has become widely used to study population structure, taxonomy, and phylogeny of animal species (Harrison 1989; Avise 1991; Simon et al. 1994). Variation in the entire mtDNA genome of five species of *Pissodes*, including the four species of the *P. strobi* group, was investigated using restriction fragment length polymorphism (RFLP) analysis (Boyce et al. 1994). Results indicated that restriction sites could provide informative characters for species-level diagnostics, although there was significant size variation and heteroplasmy in *Pissodes* mtDNA (Boyce et al. 1989). Only a few individuals from only three populations in western Canada (British Columbia) were included in this survey. To expand on this work and develop an easy-to-use and reliable diagnostic procedure to identify *Pissodes* species, we used heterologous primers and the polymerase chain reaction (PCR) to amplify a 1585-bp (base pair) region of the mtDNA that does not exhibit size variation, including half of the cytochrome oxidase I (COI) and all of the tRNA leucine and COII genes. By using endonucleases to cleave this amplified segment of mtDNA, we have assessed restriction site variation in *P. strobi*, *P. terminalis*, *P. schwarzi*,

TABLE 1. Collection localities and hosts of *Pissodes* sampled for mtDNA variation

Species	Hosts	Collection localities	Locality designation*	No. of specimens
strobi	White spruce leaders	Hay River, NWT	a	5
	White spruce leaders	Ft. Smith, NWT	d	5
	White spruce leaders	Calling L., AB	b	5
	White spruce leaders	Wabamun, AB	e	9
	White spruce leaders	Swan Hills, AB	g	5
	White spruce leaders	McDowell, SK	h	5
	White spruce leaders	Carberry, MB	c	5
	Engelmann spruce leaders	Yoho Nat. Park, BC	f	4
	Sitka spruce leaders	Kennedy L., Tofino, BC	i	12
terminalis	Lodgepole pine leaders	Watson Lake, YK	n	5
	Lodgepole pine leaders	100 Mile House, BC	m	4
	Lodgepole pine leaders	Swan Hills, AB	j	5
	Lodgepole pine leaders	Hinton, AB	l	5
	Jack pine leaders	McDowell, SK	h	3
	Jack pine leaders	Fort a la Corne, SK	k	5
schwarzi	Lodgepole pine boles	Clearwater, BC	o	5
	Lodgepole pine boles	N. of Kamloops, BC	p	5
	Lodgepole pine boles	Quartz Ck., Donald, BC	q	4
	Lodgepole pine boles	Ellis Ck., Penticton, BC	r	12
	White spruce boles	McDowell, SK	h	3
nemorensis	Slash pine logs	Gainesville, FL	s	5
	Pine logs	Syracuse, NY	t	4
affinis	White spruce boles	McDowell, SK	h	1

* Locality designations refer to mapped collection localities in Figure 3.

and *P. nemorensis* in western Canada, and evaluated the utility of using restriction sites to identify these species. A more distantly related species, *P. affinis* Randall, was similarly studied to serve as the outgroup for reconstructing relationships among species in the *P. strobi* group.

Materials and Methods

Specimen Collection and Storage. *Pissodes terminalis*, *P. strobi*, *P. schwarzi*, and *P. affinis* were collected from 18 localities in British Columbia, Alberta, Saskatchewan, Northwest Territories, and Yukon Territory (Table 1), representing the region of range overlaps for these species (Fig. 1). Adults were reared in the laboratory from infested material collected in the field. Most specimens were frozen at −80°C until used. Only one species was collected at each locality, except for McDowell, Saskatchewan, where all four species were collected in one stand. Specimens of *P. nemorensis* were obtained from New York and Florida, where adults were collected from the surface of freshly felled pine logs. All beetles were identified to species based on a combination of morphological characters, host species, damage characteristics, and phenology. For each collection site and species, 2–40 pinned voucher specimens were deposited in the reference collection at the Northern Forestry Centre. The abdomens and elytra of each specimen analyzed for mtDNA variation were stored in genitalia vials, labeled, and deposited in the same collection.

mtDNA Extraction and Amplification. Genomic DNA was extracted from 121 individual *Pissodes* specimens (Table 1), mostly males, using methods published in detail elsewhere (Langor and Sperling 1994; Sperling and Hickey 1994). This DNA was used as templates for amplification of mtDNA fragments by the PCR (Saiki et al. 1988). A 1585-bp segment of mtDNA, including half of the COI gene and all of the tRNA leucine and COII genes, was

amplified. Amplification reactions were performed in 50 μL mixtures using Taq DNA polymerase and buffer (Promega), $MgCl_2$, dNTPs (Pharmacia), two heterologous primers, DNA template, and water. Thermal cycling was done in a Perkin-Elmer-Cetus or Coy 60 programmable thermal cycler. The PCR consisted of 30–35 cycles programmed as follows: denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1.5 min.

Restriction Sites. mtDNA was digested with 10 endonucleases: *Bcl* I, *Dde* I, *Dra* I, *Hinf* I, *Msp* I, *Rsa* I, *Taq* I, *Xba* I, *Eco*R V, and *Hae* III, according to manufacturer's specifications (New England Biolabs Inc.). These enzymes were chosen because they showed differences in restriction sites between species in a preliminary survey of numerous enzymes. After digestion, fragments were separated on 0.8–1.5% agarose gels. A123-pb ladder (GIBCO BRL) was used as a size standard. Gels were stained in ethidium bromide (0.1 g/L). Restriction fragments were visible when gels were exposed to ultraviolet light, and each gel was photographed to provide a permanent record. The relative positions of all restriction sites were determined by double digestions, partial digestions, and examination of sequence data (Sperling and Langor, unpublished data). It was possible to distinguish fragments, and hence restriction sites, that differed by as little as 30 bp.

Data Analyses. Sequence divergence among mtDNA haplotypes was estimated from restriction site data using the nucleotide-counting formula of Nei and Tajima (1983). UPGMA phenograms (Sneath and Sokal 1973) were constructed using SAS (SAS Institute 1985) to compare our mtDNA data with published phenograms based on allozymes and other mtDNA data; estimated percentage sequence divergence (δ of Nei and Tajima 1983) was used as a distance measure. Haplotype and nucleotide diversity within species of *Pissodes* were calculated using formulae 8.4, 8.12, 10.5, and 10.9 of Nei (1987). Phylogenetic analyses were performed using PAUP 3.1 (Swofford 1993) by analyzing the presence and absence of restriction sites as unordered characters. *Pissodes affinis* was used as an outgroup to root the other four species. Data were submitted to 10 independent heuristic searches (each with a different randomly chosen starting seed) to identify the most parsimonious trees. The relative level of support for particular phylogenetic groupings was assessed with the bootstrap method (100 runs) (Felsenstein 1985) using a heuristic search as implemented in PAUP (Swofford 1993).

Results

Restriction Site Variation and Haplotype Distribution. Twenty-four haplotypes were found among the 121 maternal lineages surveyed, 10 for *P. strobi*, six for *P. schwarzi*, four each for *P. nemorensis* and *P. terminalis*, and one for *P. affinis* (Appendix 1). One haplotype (TA) was shared by *P. terminalis* (16 specimens) and *P. nemorensis* (one specimen from New York). No length variation or restriction site polymorphism was detected within individual specimens. A total of 47 restriction sites were detected and were distributed evenly across the entire mtDNA segment (Fig. 2); 10 sites were produced by enzymes recognizing 6-bp sequences and 37 by enzymes recognizing 4-bp sequences. Two of these sites (H3 and H4) were too close together to be distinguished without sequencing, and were thus combined (H3/4) for phylogenetic analyses. There was intraspecific polymorphism for 15 restriction sites (eight endonucleases) (Appendix 1; Fig. 2). Single haplotypes had four to six sites for 6-base enzymes and 10–19 for 4-base enzymes (Appendix 1). The total number of sites was 14–23 (mean = 17.3) representing 4.0–6.3% (mean = 5.0%) of the 1585-bp mtDNA segment.

Polymorphism was found in eight of the nine sampled populations of *P. strobi*, two of six of *P. terminalis*, three of five of *P. schwarzi*, and both populations of *P. nemorensis* (Fig. 3). In *P. strobi*, the SA haplotype was the most common (17 of 55 individuals) and widely distributed (six of nine sites). Most haplotypes exhibited a limited distribution and

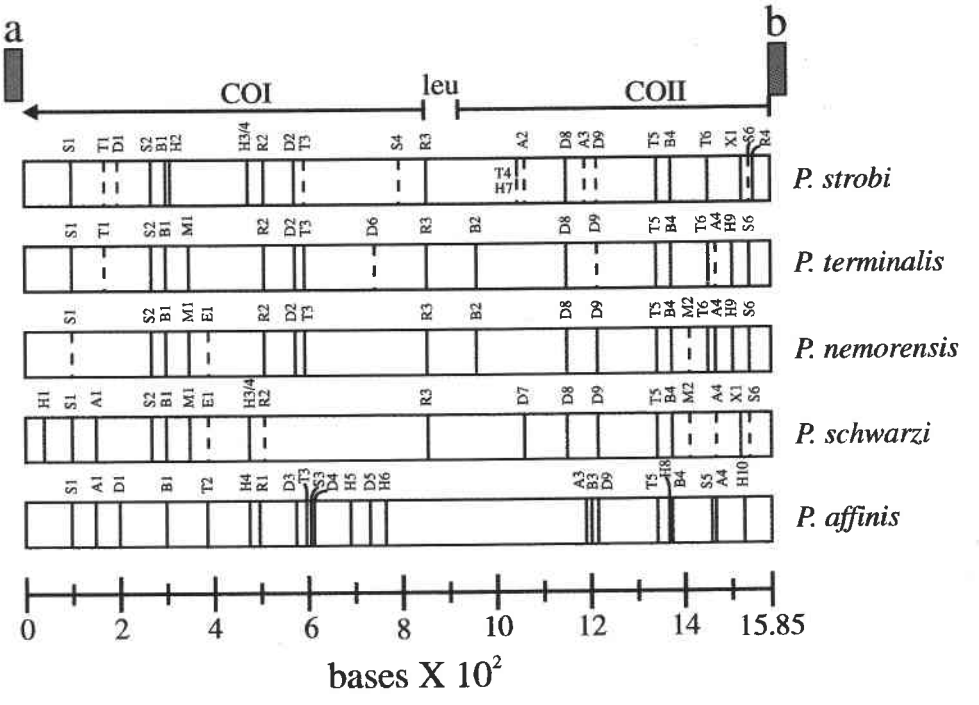

FIG. 2. Restriction site maps for a 1585-bp segment of mtDNA for five species of *Pissodes*. The map for each species is a composite of restriction sites observed in all haplotypes of that species. Broken lines indicate variable sites. Restriction enzyme abbreviations are as follows: A = *Hae* III, B = *Bcl* I, D = *Dde* I, E = *Eco*R V, H = *Hinf* I, M = *Msp* I, R = *Dra* I, S = *Rsa* I, T = *Taq* I, X = *Xba* I.

Primer **a** = 5′CAACATTTATTTTGATTTTTTGG3′

Primer **b** = 5′GAGACCATTAATTGCTTTCAGTCATCT3′

some (SC, SI, SJ) were found only at the northwestern periphery of the range of the species. A rigorous comparison of haplotype variation among host species is precluded because only two populations were sampled from hosts other than white spruce, one each from Engelmann and Sitka spruces (Table 1). Haplotype SH was collected from all three hosts but SJ was found only in Sitka spruce (Fig. 3). In *P. terminalis*, the TA haplotype was found in 16 of 27 individuals, five of six sites, and both pine species; other haplotypes were found only in populations near the periphery of the species range (Table 1; Fig. 3). In *P. schwarzi*, the WA haplotype was found in 15 of 29 specimens and three of five sites (Fig. 3). The other five haplotypes were each restricted to only one site. In *P. nemorensis*, the NA haplotype was the most common and the only haplotype found at both sites (Fig. 3).

Genetic Divergence. Three major groups of haplotypes in the *P. strobi* species group are evident when clustered (UPGMA) using estimated percentage sequence divergence as a distance measure (Fig. 4). The largest group of haplotypes, designated the S lineage, includes all of the *P. strobi* haplotypes. The haplotypes of *P. schwarzi* (W lineage) also form a distinct cluster; however, the haplotypes of *P. terminalis* and *P. nemorensis* cluster together to form the T–N lineage. Mean sequence divergence between haplotypes in the S and T–N lineages was 10.5%, and divergence between the W and S + T–N lineages was 12.5%. Species in the *P. strobi* species group were on average about 25.2% diverged from *P. affinis*.

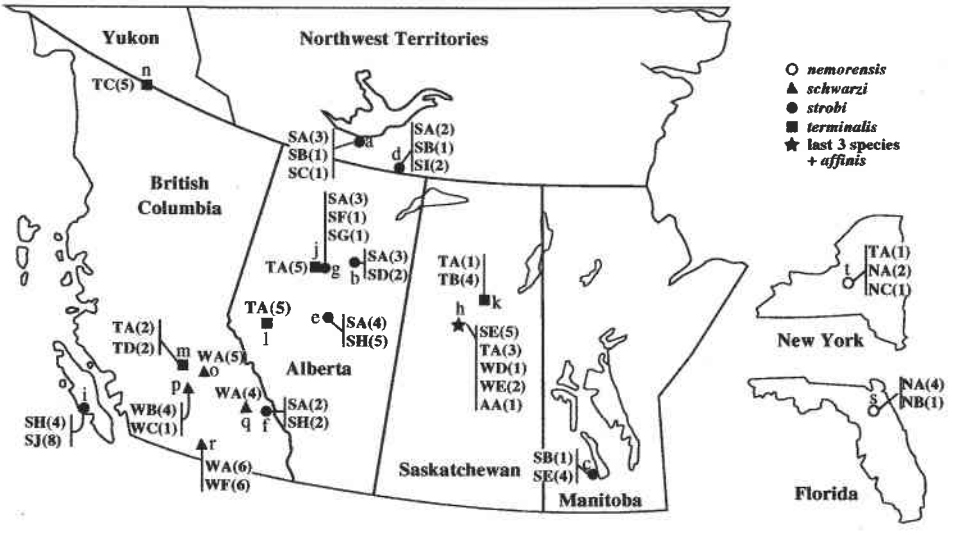

FIG. 3. Distribution of mtDNA haplotypes of five species of *Pissodes* in western Canada. The numbers of specimens are indicated in parentheses. The lowercase letters refer to collection localities listed in Table 1.

Genetic diversity within the four species of the *P. strobi* group varied considerably (Table 2). The highest haplotype diversity was observed in *P. strobi* and the highest nucleotide diversity in *P. schwarzi*. The lowest haplotype and nucleotide diversity was observed in *P. nemorensis*. Haplotype diversity in *P. strobi* was significantly higher than in other species; however, there were no statistical differences among lineages with respect to nucleotide diversity because of high variance, mainly a result of the stochasticity of the evolutionary process (Nei 1987).

Phylogenetic Relationships. A phylogenetic analysis of restriction sites as unordered characters was performed using the heuristic search algorithm in PAUP 3.1. Ten independent (in terms of starting point) searches each yielded the same set of 19 equally parsimonious trees (60 steps) for the 24 haplotypes. The consistency index of these trees was 0.60, excluding uninformative characters. The trees differed from each other in the arrangement of the SA, SC, SD, SF, SG, and SJ haplotypes within the S lineage. A parsimony network of the strict consensus tree is illustrated in Figure 5. The monophyly of *P. strobi* haplotypes was supported by 96% of the bootstrap replicates, 96% support the monophyly of *P. terminalis* and *P. nemorensis*, and 81% support the monophyly of *P. terminalis*, *P. nemorensis*, and *P. strobi* (Fig. 5).

Discussion

Haplotype Diversity and Gene Flow. Quantity and pattern of intraspecific genetic variation are determined by factors such as population structure, gene flow, extinction, and selection (Avise et al. 1987; Harrison 1991). The differences in haplotype diversity among the four *Pissodes* spp. (Table 2) may be partly explained by these factors; however, we believe that the higher haplotype diversity in *P. strobi* may be largely explained by the fact that more individuals and populations of this species over a larger geographic area were examined than of the other three species. Haplotype diversity for *P. strobi*, excluding geographically outlying populations (a, c, d, and i) (Fig. 3), is 0.718 ± 0.058, about 12% lower than when these populations are included (Table 2).

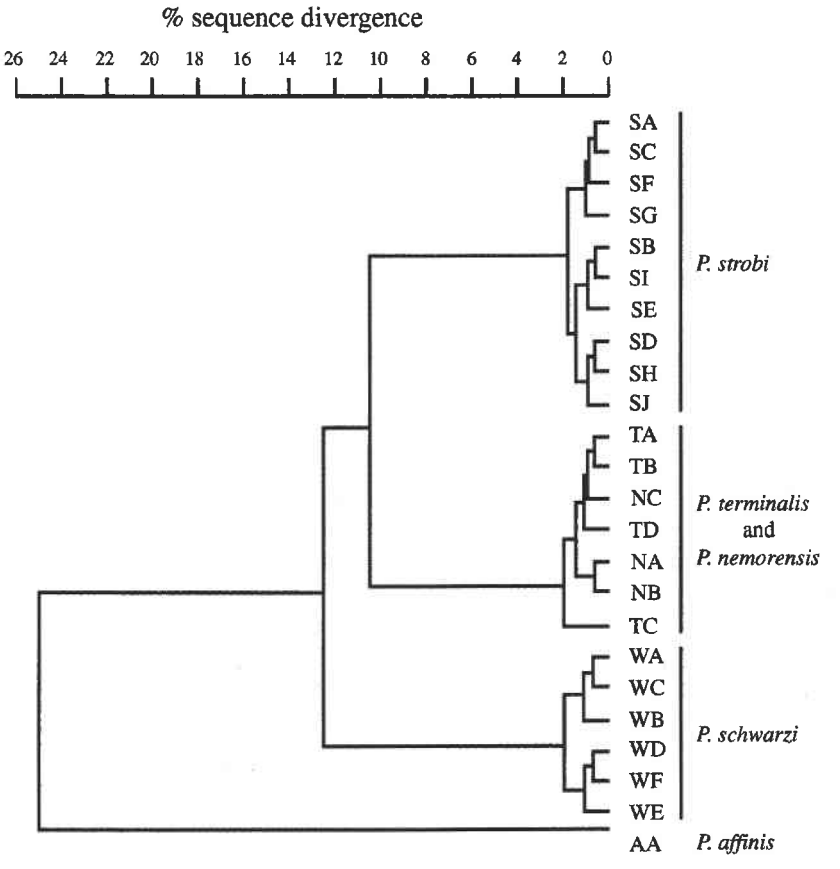

FIG. 4. UPGMA phenogram of percentage sequence divergence (δ of Nei and Tajima 1983) among mtDNA haplotypes.

Geographic patterns of intraspecific variation in haplotypes are similar for all four species in the *P. strobi* group; one haplotype is relatively widespread throughout most of the sampled range and the other haplotypes are relatively localized, usually near the margins of the range of the species (Fig. 3). This resembles a Category V mtDNA phylogeographic pattern (Avise et al. 1987). Such a pattern is anticipated in species with historically intermediate levels of gene flow between populations, where presumed ancestral genotypes occur over a wide area but new genotypes have not yet spread very far. The fact that many divergent haplotypes tend to occur near the edge of the species range, e.g. SB, SC, SI, and SJ of *P. strobi*, suggests isolation of peripheral populations.

Interestingly, Boyce et al. (1994) also saw a Category V phylogeographic pattern for *P. terminalis* and *P. nemorensis*; however, the pattern for *P. strobi* was similar to Category III. The fact that 39 of 40 *P. strobi* haplotypes found by Boyce et al. (1994) were each restricted to only one locality suggested historically limited gene flow between populations not subdivided by firm long-term geographic barriers to dispersal. Boyce et al. (1994) sampled only one population of *P. strobi* in western Canada (British Columbia). Therefore, it is possible that there are different levels of gene flow in different parts of the range of this species; however, this possibility is tempered by the fact that we studied only

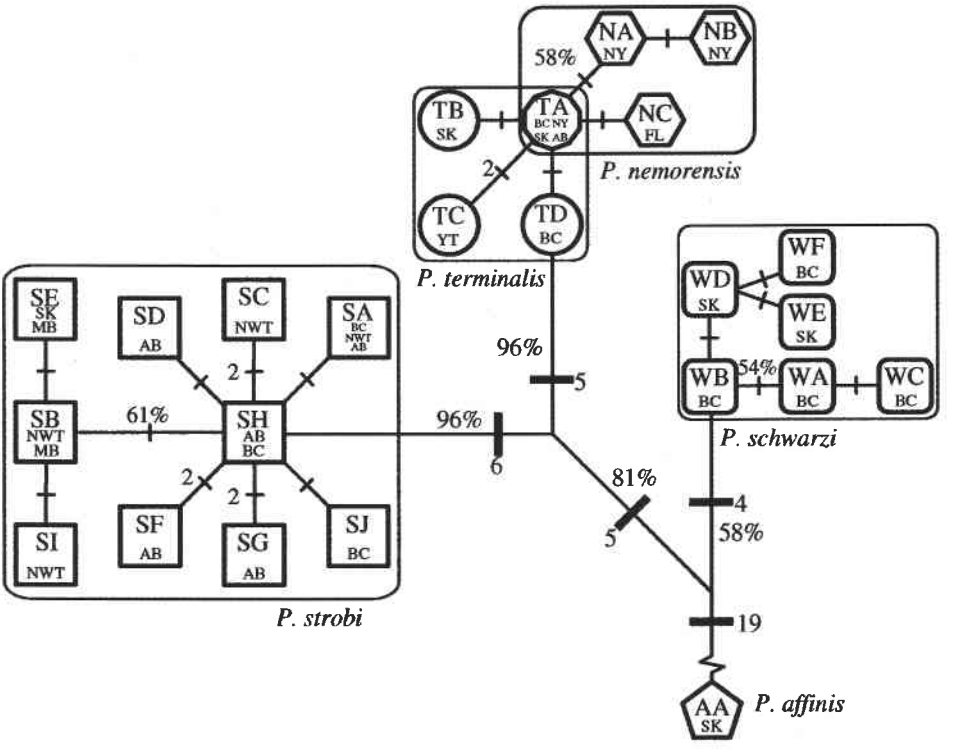

FIG. 5. Parsimony network of mtDNA haplotypes. Large uppercase letters refer to haplotypes of specimens surveyed (listed in Appendix 1). Numbers and single bars on branches refer to the number of apomorphies. The small uppercase letters refer to localities where haplotypes were found. Numbers expressed as percentages refer to extent of support found in 100 bootstrap runs; only branches supported by ≥ 50% are indicated. Codes for states and provinces are as follows: AB = Alberta, BC = British Columbia, FL = Florida, NWT = Northwest Territories, NY = New York, MB = Manitoba, SK = Saskatchewan, YT = Yukon Territory.

a portion of the mtDNA studied by Boyce. Results may not be comparable between the two studies if evolution differs significantly between COI/COII and the rest of the mtDNA.

Sequence Divergence. The mtDNA of *Pissodes* is unusual in that the genome is relatively large (30–36 kb) and exhibits a high degree of heteroplasmy (Boyce et al. 1989). Additionally, the mtDNA of the four closely related species of the *P. strobi* group exhibit an unusually high amount of intra- and inter-specific sequence divergence, as estimated from restriction site data (Fig. 4; Boyce et al. 1994). The interspecific sequence divergences reported by

TABLE 2. Genetic diversity within species of the *Pissodes strobi* group

Species	Sample size	Haplotype diversity* (h ± 2SE)	Nucleotide diversity† (π ± 2SE)
strobi	55	0.817±0.018	0.0102±0.0086
terminalis	27	0.598±0.107	0.0067±0.0065
schwarzi	29	0.675±0.072	0.0111±0.0100
nemorensis	9	0.549±0.254	0.0051±0.0048

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

TABLE 3. Fragment sizes produced when amplified mtDNA of the four species of the *Pissodes strobi* species group is digested by 10 restriction enzymes. For *Hinf* I, some fragments are approximate ±6 bp as a result of the possible presence of two restriction sites in close proximity

Restriction enzyme	*Pissodes* species*	Restriction sites†	Fragment sizes (bp)‡
Bcl I	s,w	B1, B4	237, 325, 1074
	t,n	B1, B2, B4	237, 325, 415, 659
Dra I	s	R2, R3, R4	67, 341, 534, 694
	t,w,n	R2, R3	341, 534, 761
	w	R3	761, 875
Xba I	t,n		1636
	s,w	X1	94, 1542
*Eco*R V	s,t,w,n		1636
	w,n	E1	397, 1239
Hinf I	s	H2, H3/4, H7	335, ~153, 566, ~582
	s	H2, H3/4	335, ~153, ~1148
	t,n	H9	110, 1526
	w	H1, H3/4	56, ~432, ~1148
Msp I	s		1636
	t,w,n	M1	368, 1268
	w,n	M1, M2	197, 368, 1071
Taq I	s	T4, T5, T6	111, 163, 294, 1068
	s	T3, T4, T5, T6	111, 163, 294, 450, 618
	s	T1, T4, T5, T6	111, 163, 197, 294, 871
	t,n	T3, T5, T6	111, 163, 618, 744
	t	T1, T3, T5, T6	111, 163, 197, 421, 744
	w	T5	274, 1362
Rsa I	s,t,w,n	S1, S2, S6	80, 115, 180, 1261
	s	S1, S2, S4, S6	80, 115, 180, 523, 738
	s	S1, S2, S4	115, 180, 523, 818
	w	S1, S2	115, 180, 1341
	n	S2, S6	80, 295, 1261
Dde I	s	D1, D2, D8, D9	72, 225, 372, 394, 573
	s	D1, D2, D8	225, 372, 466, 573
	s,t	D2, D8	466, 573, 597
	t,n	D2, D8, D9	72, 394, 573, 597
	t	D2, D6, D8, D9	72, 165, 394, 408, 597
	w	D7, D8, D9	72, 80, 394, 1090
Hae III	s,t		1636
	s	A3	423, 1213
	s	A2	552, 1084
	t,n	A4	144, 1492
	w	A1	171, 1465
	w	A1, A4	144, 171, 1321

* s =*P. strobi*, t = *P. terminalis*, n = *P. nemorensis*, w = *P. schwarzi*.
† Restriction site designations correspond to those in Figure 2.
‡ Sizes of end fragments include the attached primers (Fig. 2).

Boyce et al. (1994) are generally higher than those estimated in this study, presumably because the COI and COII regions are more conserved than other regions included in the earlier study.

In the current study, sequence divergence is overestimated because choice of endonucleases was biased to include only those that showed restriction site differences between the species in a preliminary survey of many enzymes. Choice of restriction enzymes should be unbiased to obtain a reasonable estimate of sequence divergence from restriction sites.

Actual interspecific sequence divergences in the COI and COII regions averaged around 40% lower than those estimated from restriction enzymes (Sperling and Langor, unpublished data). Even when estimated sequence divergences (Fig. 4) are reduced by 40%, they are still higher than those reported for most other groups of closely related insect species (e.g. Martin and Simon 1990; Brower and Boyce 1991; Willis et al. 1992; Beckenbach et al. 1993; Sperling 1993*a*, 1993*b*; Vogler et al. 1993; Sperling and Harrison 1994; Sperling and Hickey 1994, 1995). The high level of mtDNA sequence divergence among species in the *P. strobi* group contrasts greatly with the lower levels of allozyme and morphological divergence (Hopkins 1911; Phillips 1984; Langor, unpublished). This suggests substantially different rates of evolution between the nuclear and mitchondrial genomes. Boyce et al. (1994) suggest that the extensive polymorphism for restriction sites and size in mtDNA indicates an accelerated evolution in *Pissodes* mtDNA, compared with that of other insects.

Species Relationships. A phylogenetic analysis of restriction sites in the COI and COII region revealed that *P. terminalis* and *P. nemorensis* are most closely related (Figs. 5, 6). This agrees with the findings of Boyce et al. (1994), based on a RFLP analysis of the entire coding region; however, the two phylogenies differed in that we found that *P. strobi* was most closely related to the *terminalis-nemorensis* clade, whereas Boyce et al. (1994) found that *P. schwarzi* was the closest relative. The differences between the two molecular phylogenies are likely related to differences in choice of endonucleases (only four of 10 were common to both studies) and the portion of the mtDNA molecule targeted for study. Also, both molecular phylogenies conflict with hypothesized relationships based on allozyme variation; Phillips (1984) found that *P. nemorensis* was more closely related to *P. strobi* than to *P. terminalis*; allozyme variation in *P. schwarzi* was not investigated. Smith and Takenouchi (1962) present a different view of relationships among species; they suggest that *P. terminalis* is a hybrid species formed from introgression between *P. yosemiti* Hopkins (a junior synonym of *P. schwarzi*) and likely *P. strobi*. Molecular genetics data do not support such a hybrid origin for *P. terminalis*. Hopkins' (1911) classification of species based on morphology suggests that he thought that *P. nemorensis* and *P. schwarzi* were most closely related; he placed these two species and their currently recognized junior synonyms in Series c4. Hopkins did not recognize *P. terminalis*. Thus, it is clear that there still is no consensus regarding relationships among species in the *P. strobi* group. Resolution of relationships among these species will require a thorough phylogenetic analysis of morphology (Langor, unpublished data), DNA (nuclear and mitochondrial) characters, cytogenetic characters, and ecological traits.

Based on cytogenetic evidence, Smith and Sugden (1969) synonymized *P. sitchensis* Hopkins, a pest of Sitka spruce, and *P. engelmanni* Hopkins, a pest of Engelmann spruce, with *P. strobi*, a pest of eastern white pine and other spruces. Morphological characters also seem to support this synonymy (Langor, unpublished data). We examined mtDNA in one population of *P. strobi* from Sitka spruce, one from Engelmann spruce, and eight from white spruce. There was no evidence to suggest that these populations were not conspecific. The population from Sitka spruce (population i, Fig. 3) is composed mainly of the SJ haplotype, which is not found in any other population of *P. strobi*. However, these data are not sufficient to distinguish between the effects of geographic isolation and host species on reducing gene flow. A larger survey is underway to assay mtDNA variation in *P. strobi* among host species.

Diagnostics. All restriction enzymes except *Eco*R V provide information of diagnostic value (Table 3), but no single enzyme was sufficient to discriminate among all species. The three most diagnostically informative enzymes are *Bcl* I, *Dra* I, and *Hinf* I, and we recommend that these be used for mtDNA-based identification of species in the *P. strobi* group. Each of these three enzymes cut the mtDNA fragment of each species at least once (Table 3; Fig. 6), and thereby provides an internal assay for enzyme activity. Five haplotypes were observed using a combination of all three enzymes, two for *P. strobi* (Bc.a + Dr.a + Hi.a, Bc.a + Dr.a +

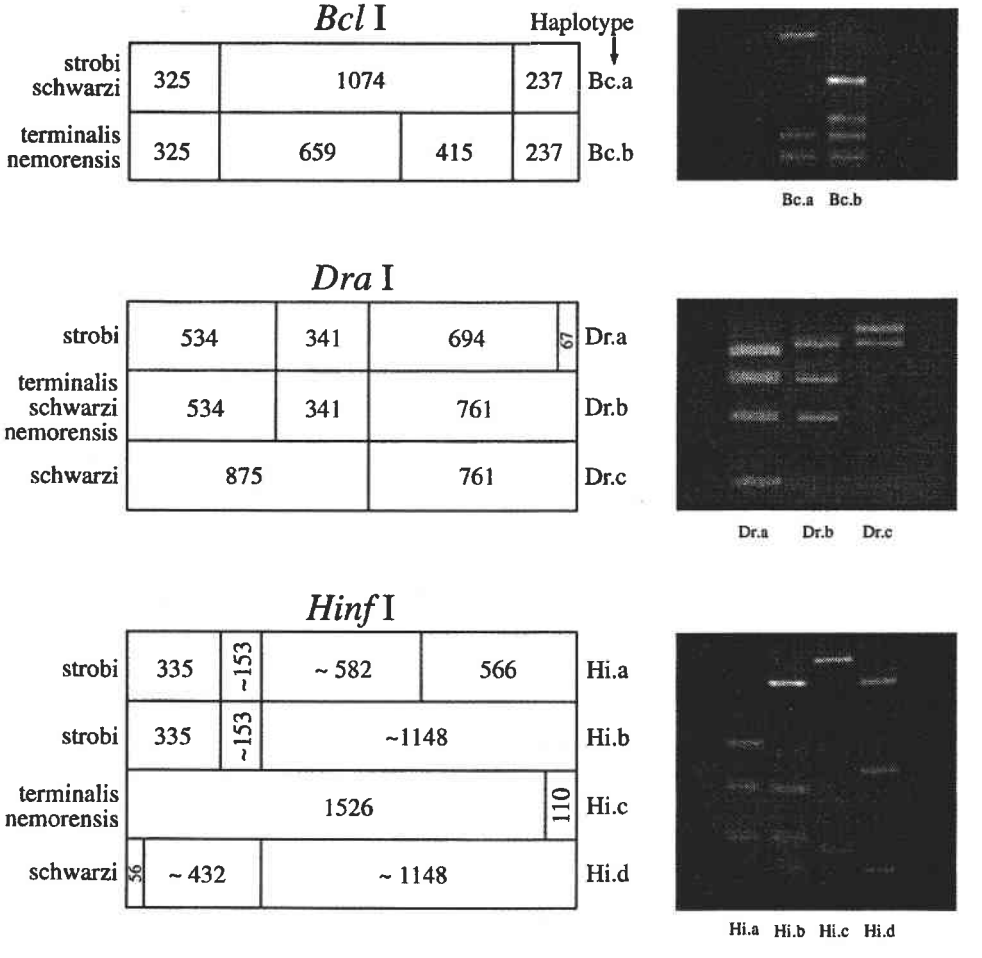

FIG. 6. Fragments produced by digestion of the amplified fragment with three restriction enzymes, *Bcl* I, *Dra* I, and *Hinf* I. The size of the terminal fragments includes the appropriate primer. For *Hinf* I, some fragments are approximate ± 6 bp due to the possible presence of two restriction sites in close proximity.

Hi.b), two for *P. schwarzi* (Bc.a + Dr.b + Hi.d, Bc.a + Dr.c + Hi.d), and one shared by *P. terminalis* and *P. nemorensis* (Bc.b + Dr.b + Hi.c). Except for *P. terminalis* and *P. nemorensis*, each pair of species is distinguishable by the presence or absence of at least three restriction sites (Fig. 6). It may be difficult to distinguish among *P. terminalis* and *P. nemorensis* haplotypes where the ranges of these species apparently overlap in Manitoba. Although *P. canadensis* Hopkins (junior synonym of *P. nemorensis*) was reported from central Manitoba (Hopkins 1911; Manna and Smith 1959), we have not yet seen these specimens to confirm their identity. Also, in parts of the eastern United States where *P. strobi* and *P. nemorensis* may hybridize naturally (Boyce et al. 1994), mtDNA characters are of limited use for distinguishing among these species; however, hybridization among species in the *P. strobi* group has not been detected in western Canada.

At this time, the DNA-based approach to diagnostics of the *P. strobi* species group is more reliable than morphological and allozyme characters. Using mtDNA characters it is

possible to discriminate reliably among the three major mtDNA lineages, i.e. *strobi*, *schwarzi*, and *terminalis-nemorensis*, in western Canada. DNA diagnostic characters are also attractive because they are not limited to a single developmental state (e.g. Sperling et al. 1994). This allows for easy identification of larvae and pupae and association of immature stages with adults. Also, assessment of DNA characters is relatively unconstrained by the state of preservation of samples, although fresh material yields the best results. In contrast, cytogenetic characters, although taxonomically informative for *Pissodes* species (Smith and Virkki 1978), can only be assessed in fresh material. We successfully amplified the entire 1585-bp mtDNA segment from one specimen of *P. terminalis* that was preserved in 98% ethanol for 30 days; however, it is unusual to amplify successfully segments of this size from preserved material. In more poorly preserved material, DNA can still be amplified and identified in smaller fragments using primers that anneal in closer proximity and amplify across diagnostically informative regions (e.g. Sperling et al. 1994). We have developed several primers for *Pissodes* that allow for amplification of the entire 1585-bp segment in smaller fragments. Small segments of insect DNA have also been amplified and sequenced from dried museum material and fossils (e.g. Cano et al. 1993). We hope to use these techniques to amplify DNA from dried *Pissodes* specimens to help resolve morphological ambiguities.

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Appendix 1. Restriction sites for 24 mtDNA halotypes of *Pissodes*.

			Restriction sites																		
			H1	S1	A1	T1	D1	S2	B1	H2	M1	T2	E1	H3/4	R1	R2	D2	D3	T3	S3	D4	H5
Species	Haplotype	*N*	33	92	148	174	202	272	302	312	345	372	374	≈ 465	496	511	574	581	595	601	604	675
strobi	SA	17	0	1	0	0	1	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0
	SB	3	0	1	0	0	1	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0
	SC	1	0	1	0	0	1	1	1	1	0	0	0	1	0	1	1	0	1	0	0	0
	SD	2	0	1	0	1	1	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0
	SE	9	0	1	0	0	1	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0
	SF	1	0	1	0	0	1	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0
	SG	1	0	1	0	0	0	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0
	SH	11	0	1	0	0	1	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0
	SI	2	0	1	0	0	1	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0
	SJ	8	0	1	0	0	1	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0
schwarzi	WA	15	1	1	1	0	0	1	1	0	1	0	1	1	0	0	0	0	0	0	0	0
	WB	4	1	1	1	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0
	WC	1	1	1	1	0	0	1	1	0	1	0	1	1	0	0	0	0	0	0	0	0
	WD	1	1	1	1	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0
	WE	2	1	1	1	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0
	WF	6	1	1	1	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0
terminalis	TA	17*	0	1	0	0	0	1	1	0	1	0	0	0	0	1	1	0	1	0	0	0
	TB	4	0	1	0	0	0	1	1	0	1	0	0	0	0	1	1	0	1	0	0	0
	TC	5	0	1	0	1	0	1	1	0	1	0	0	0	0	1	1	0	1	0	0	0
	TD	2	0	1	0	0	0	1	1	0	1	0	0	0	0	1	1	0	1	0	0	0
nemorensis	NA	6	0	1	0	0	0	1	1	0	1	0	1	0	0	1	1	0	1	0	0	0
	NB	1	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	1	0	0	0
	NC	1	0	1	0	0	0	1	1	0	1	0	0	0	0	1	1	0	1	0	0	0
affinis	AA	1	0	1	1	0	1	0	1	0	0	1	0	1	1	0	0	1	1	1	1	1

* Includes one specimen of *P. nemorensis* from Syracuse, NY.

Site designations and map positions correspond with those in Figure 2

and map positions

D5	D6	H6	S4	R3	B2	T4	H7	A2	D7	D8	A3	B3	D9	T5	H8	B4	M2	T6	S5	A4	H9	X1	H10	S6	R4
733	739	765	796	852	961	1045	1047	1061	1067	1147	1190	1205	1219	1339	1389	1376	1415	1450	1452	1469	1503	1519	1527	1533	1546
0	0	0	0	1	0	1	1	0	0	1	0	0	0	1	0	1	0	1	0	0	0	1	0	1	1
0	0	0	1	1	0	1	0	0	0	1	0	0	0	1	0	1	0	1	0	0	0	1	0	1	1
0	0	0	0	1	0	1	1	0	0	1	0	0	0	1	0	1	0	1	0	0	0	1	0	1	1
0	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0	1	0	1	0	0	0	1	0	1	1
0	0	0	1	1	0	1	0	0	0	1	0	0	0	1	0	1	0	1	0	0	0	1	0	0	1
0	0	0	0	1	0	1	1	0	0	1	0	0	1	1	0	1	0	1	0	0	0	1	0	1	1
0	0	0	0	1	0	1	1	0	0	1	0	0	0	1	0	1	0	1	0	0	0	1	0	1	1
0	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0	1	0	1	0	0	0	1	0	1	1
0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	0	1	0	1	0	0	0	1	0	1	1
0	0	0	0	1	0	1	0	1	0	1	0	0	0	1	0	1	0	1	0	0	0	1	0	1	1
0	0	0	0	1	0	0	0	0	1	1	0	0	1	1	0	1	0	0	0	0	0	1	0	0	0
0	0	0	0	1	0	0	0	0	1	1	0	0	1	1	0	1	0	0	0	0	0	1	0	0	0
0	0	0	0	1	0	0	0	0	1	1	0	0	1	1	0	1	0	0	0	0	0	1	0	1	0
0	0	0	0	1	0	0	0	0	1	1	0	0	1	1	0	1	1	0	0	0	0	1	0	0	0
0	0	0	0	1	0	0	0	0	1	1	0	0	1	1	0	1	1	0	0	1	0	1	0	0	0
0	0	0	0	1	0	0	0	0	1	1	0	0	1	1	0	1	1	0	0	0	0	1	0	0	0
0	0	0	0	1	1	0	0	0	0	1	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0
0	1	0	0	1	1	0	0	0	0	1	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0
0	0	0	0	1	1	0	0	0	0	1	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0
0	0	0	0	1	1	0	0	0	0	1	0	0	1	1	0	1	0	1	0	0	1	0	0	1	0
0	0	0	0	1	1	0	0	0	0	1	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0
0	0	0	0	1	1	0	0	0	0	1	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0
0	0	0	0	1	1	0	0	0	0	1	0	0	1	1	0	1	1	1	0	1	1	0	0	1	0
1	0	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	1	1	0	0	1	0	0