Genetic Relatedness of North American Populations of *Tomicus piniperda* (Coleoptera: Scolytidae)

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ABSTRACT We used DNA fingerprinting by random amplified polymorphic (RAPD) DNA and electrophoretic characterization of esterase isozymes to investigate the genetic relatedness of North American populations of the exotic bark beetle *Tomicus piniperda* (L.). Cluster analyses of genetic distances among populations identified the Illinois population as an outlier population with mean genetic distances to other populations averaging 0.895 (where complete dissimilarity = 2), compared with genetic distance averages of 0.595 among populations excluding Illinois. When genetic distance means and geographical distance between populations were compared, the results suggested that *T. piniperda* populations in the United States were established separately in Illinois near Lake Michigan and in Ohio along Lake Erie. Molecular markers indicated that insects derived from the 2 founder groups were interbreeding in contiguous regions in western Indiana.

KEY WORDS *Tomicus piniperda*, random amplified polymorphic DNA, genetic variation, exotic insects

(L.) is an exotic pest of pines (*Pinus spp.*) in North America that is native to Europe, Asia, and North Africa (USDA 1972). An established population of this beetle was discovered in a Christmas tree plantation in Lorain County, Ohio, in June 1992 (Haack and Lawrence 1995). After the initial discovery, surveys throughout the Great Lakes region indicated the presence of *T. piniperda* in 42 counties in 6 states by the end of 1992. By August 1996, *T. piniperda* had been detected in 170 counties in 8 states in the United States and in 14 counties in Ontario, Canada. However, most of the new county records added since 1992 probably resulted from more intensive surveys rather than rapid spread by the beetle (Haack and Lawrence 1994).

Damage to host trees occurs primarily by growth loss that results from adult tunneling and feeding inside shoots; in China, however, *T. piniperda* beetles may kill living pine trees (Ye 1991). A federal quarantine has been implemented by USDA–APHIS (Animal and Plant Health Inspection Service). The quarantine restricts movement of infested pine material including logs, nursery stock, and Christmas trees, thereby causing a substantial economic impact on affected businesses.

Because *T. piniperda* is not likely to be eradicated from North America (Haack and Lawrence 1995), managers must now deal with this species by developing appropriate management strategies. The probable origin(s) and genetic relatedness of current *T. piniperda* populations should also be determined to help predict species behavior in North America and to direct future control efforts (Prokopy and Croft 1994).

Tomicus piniperda populations in the Great Lakes region originated from founder insects of unknown numbers and origins that represent a fraction of the genetic diversity of source population(s). Genetic constriction resulting from immigration and geographical isolation is often followed by a burst of expanding diversity as the immigrants adapt to and exploit their new environment and increase in numbers (Templeton 1982). If the time-lag between an event that affects evolution (such as immigration) and collection of data for analysis is too great, the probability of producing accurate phylogeny reconstructions is significantly lowered (Hillis et al. 1994). Therefore, to determine the lineages of the Great Lakes populations of *T. piniperdu,* it is important to study the populations soon after their discovery. In addition, use of a method of character analysis with sufficient resolution is necessary to detect relatively minute

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changes implicit in short-term evolution (Hillis et al. 1994). Molecular methods are appropriate for studies of short-term evolutionary change because the resolution of variation is at the nucleotide level, and changes in nucleotide sequences can be assessed without the confounding effects of environmentally or developmentally induced variability (Strauss et al. 1992).

In this study, we used random amplified polymorphic (RAPD) DNA methods for polymerase chain reaction (PCR) amplification of *T. piniperdu* DNA. RAPD-PCR, initially described by Welsh and McClelland (1990) and Williams et al. (1990), has been used extensively to assess genomic variability in a wide range of organisms including insects (Black et al. 1992, Roehrdanz and Flanders 1993, Cognato et al. 1995). We used a variation of standard RAPD-PCR methods that is based on DNA bulking (Michelmore et al. 1991, Hadrys et al. 1993, Liu et al. 1994) to fingerprint *T. piniperdu* populations. In the bulking strategy, several individuals from a population are pooled for DNA extraction and production of multiple-genome DNA. Consensus fingerprints are produced because RAPD markers that are individual-specific, or are present in <IO-20% of the bulked population, are generally poorly amplified and are not scored in agarose gels stained with ethidium bromide (Michelmore et al. 1991, Tinker et al. 1994). With this technique, use of large numbers of markers in the experimental design are practical because organisms are not treated individually. Large numbers of markers increase the likelihood that more of the genome is represented (Skroch et al. 1993) and assumptions that might be required in analyses—such as independence of characters and constancy of mutation rates (Strauss et al. 1992)—are therefore more fully supported.

To reference the RAPD-PCR data with a more traditional approach, we included a study of esterase isozymes in *T. piniperda.* Electrophoretic analysis of esterases has been a successful approach to detect variation within and among populations (Healy et al. 1991). Based on the results of these 2 approaches, we describe the genetic relatedness of *T. piniperda* subpopulations that were collected in the Great Lakes region in 1993.

Materials and Methods

Collections. One to 4 *Pinus sylvestris* L. logs were placed in 8 infested sites in 5 states in the Great Lakes region during February and March 1993: 1 site in Illinois (population IL, Will County), 2 in Indiana (population IN1, Laporte County; population IN2, Steuben County), 2 in Michigan (population MI1, Kalamazoo County; population M12, Ingham County), 1 in Ohio (population OH, Lorain County), and 2 in New York (population NY1, Niagara County; population NY2, Niagrara County) (Fig. 1). The Ohio site was in the same county where *T. piniperda* originally was discovered. The logs were subsequently infested in March and April 1993 by local populations of *T. piniperda.* In May 1993, the infested logs were returned to a laboratory in Michigan and placed individually inside labeled containers. Emergent F_1 adults were collected during several days, pooled by site, and stored frozen at -80°C. Typically, 1 adult male and 1 adult female construct each gallery (Haack and Lawrence 1995). Therefore, after the beetles had emerged, the number of galleries per log was determined to provide an estimate of the number of mating pairs that had reproduced in each log. Details of collection and sampling are given in Table 1.

Sample Bulking and DNA Extraction. By our rationale, the more individuals that are bulked from a population, the more conservative the RAPD fingerprint will be because individual variation is diluted relative to the amount of redundancy in the bulked DNA (see Tinker et al. 1994). We bulked 15 insects per population to obtain a balance of conserved and moderately variable characters for detection by RAPD-PCR. The beetles, randomly selected from each population, were ground in liquid N_2. Each bulked sample was incubated at 57°C for 1 h in 500 μl of extraction buffer (consisting of 5 mM ascorbic acid, 1.4 M NaCl, 20 mM EDTA, 2% [wt:vol] cetyltrimethylammonium bromide [CTAB], and 100 mM Tris [pH 7.5]) with 70 μg of DNAse-free RNAase. Samples were extracted with chloroform and centrifuged to separate phases. The aqueous phase was collected and 0.1 times volume of 5 M potassium acetate and 2 times volume of cold, 100% ethanol were added. After holding at -20°C overnight, DNA pellets were obtained by centrifugation at 5,000 X g (5 min), rinsed in 70% ethanol, dried briefly, and subsequently dissolved in 50–100 μl 25 mM Tris (HCl) buffer.

Quantification was by microassay fluorometry (Hoeffer, San Francisco, CA). Individual insects were processed similarly, except that each insect was ground directly in the extraction buffer and quantification was by dot assay (Sambrook et al. 1989) with undiluted samples. The quality of DNA of both bulked and individual samples was evaluated by electrophoresis and ethidium bromide staining. All samples used in the study were of high quality for PCR, as demonstrated by high molecular weight DNA and no indication of RNA.

RAPD-PCR Analysis. Procedures were used to prevent contamination of PCR reactions with errant DNA (Sambrook et al 1989). Reactions were done in thin-walled, selfcapped tubes (0.2 ml) with 15.6 μl sterile water, 2.5 μl 10X Taq buffer with Mg^{++} (Boehringer Mannheim, Indianapolis, IN), 0.5 μl 2′-deoxynucleoside 5′-triphosphate mix (Boehringer Mannheim), 0.4 μl Taq enzyme (2 units, Boehringer Mannheim), 1.0 μl primer (15 pmoles, Operon, Alameda, CA), and 5 μl of 2 ng/μl DNA in water or 10 mM Tris, (pH 7.5). Amplification was conducted in an MJ Research (Water-

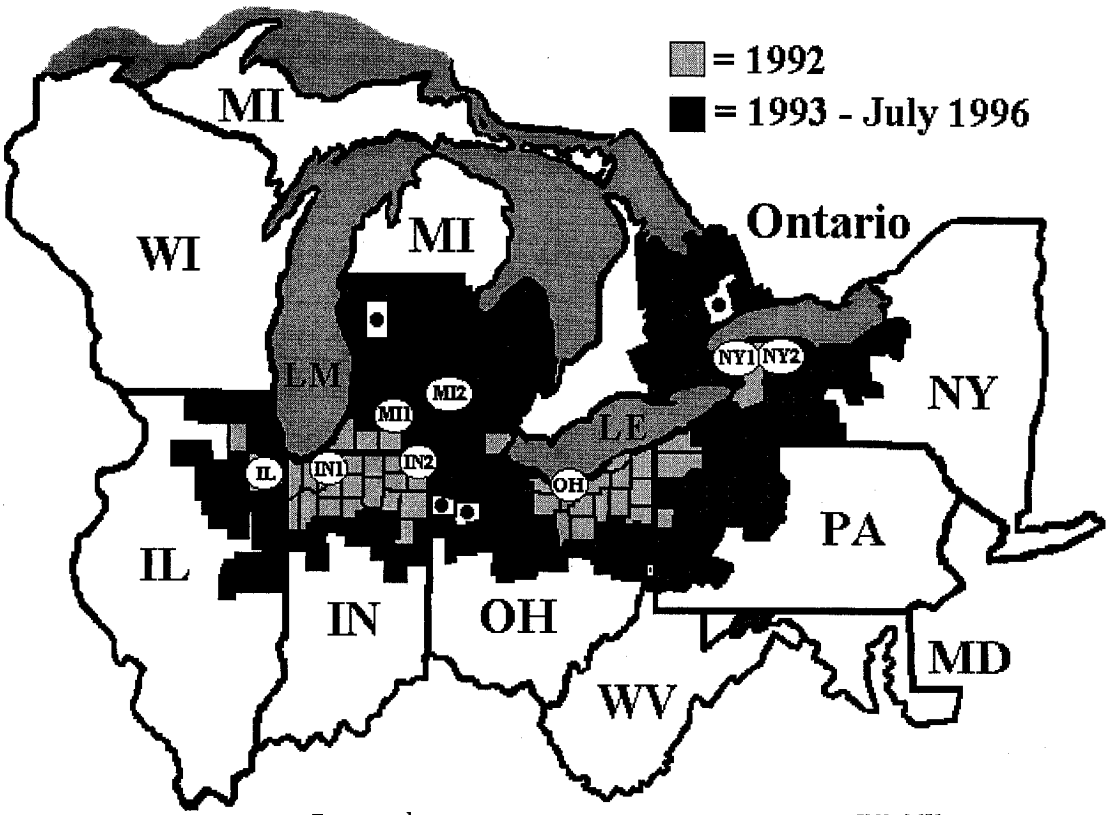

Fig. 1. Map showing known *T. piniperda* range in North America and sample sites IL, IN1, MI1, IN2, M12, OH, NY1, and NY2 of this study. The black shaded counties indicate detection as of December 1992. The grey shaded counties indicate additional detections as of July 1996. Dots indicate counties within range where no beetles have been found. LM, Lake. Michigan; LE, Lake. Erie.

town, MA) Hot Bonnet Thermocycler with the following program: step 1 (94°C for 20 s), step 2 (94°C for 5 s), step 3 (40°C for 1 min), step 4 (72°C for 2 min), step 5 (repeat steps 24, 45 times), step 6 (72°C for 2 min). After completion of step 6, samples were held at 4°C. Amplified fragments were electrophoresed in 2% agarose gels buffered in 1X TAE (Sambrook et al. 1989), stained in ethidium bromide, destained in water, and photographed with UV light. Reactions were repeated for scoring clarification.

Table 1. Sampling data for T. *piniperda* populations

Site	No. logs sampled	Total no. galleries in logs	No. F_1 adults collected	No. insects used in analyses	
				Isozyme[a]	RAPD[b]
IL1	1	26	61	26	15
IN1	4	99	152	39	15
IN2	3	81	140	29	14
MI1	4	144	146	42	15
MI2	2	97	121	38	15
OH	1	57	110	37	15
NY1	2	8	40	25	15
NY2	3	24	57	20	15

See Fig. 1 for collection sites
[a] Individual insects.
[b] Bulked insects.

Isozyme Study. Individual insects (2042 per site, Table 1) were ground in 150 μl of sample buffer (0.125 M Tris/HCl (pH 6.8), 1% Triton X-100, 25% sucrose (wt:vol), 0.01% 2-mercaptoethanol, and 0.1% bromophenol blue) and centrifuged at 10,000 X g (10 min) to remove particulate matter. Nondenaturing vertical polyacrylamide slab gel electrophoresis was done as described by Vernick et al. (1988) in a water-cooled minigel unit (Hoeffer).

After electrophoresis, the gels were incubated in the dark for 10 min in 100 ml of 0.1M sodium phosphate buffer (pH 6.8), and 0.0005% 2-mercaptoethanol, with 40 mg of naphtyl acetate (Healy et al. 1991); they were subsequently stained with 2 ml of a 5% (wt:vol) aqueous solution of fast blue BB. Gels were destained and fixed in an aqueous solution containing 10% acetic acid and 35% methanol, then air-dried between transparent cellophane membranes.

Data Analysis: RAPD-PCR. Although binary scoring (i.e., presence/absence) is most frequently used in RAPD-PCR investigations (e.g., Yu and Pauls 1995), some investigators have used multistate scoring to recognize differences in band intensity among samples (Strongman and MacKay 1993, Wolff and Peters-van Rijn 1993, Howell and

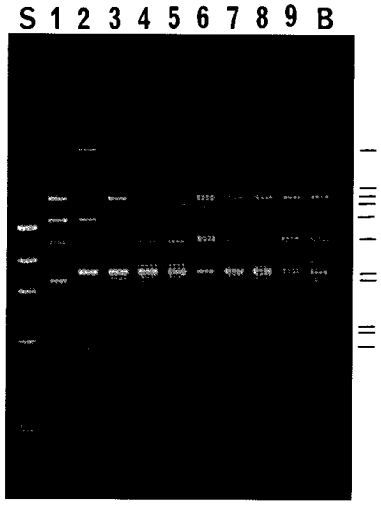

Fig. 2. DNA fingerprints of individual and bulked *T. piniperda* from population OH produced by RAPD-PCR using primer B8. Lanes 1–9, individual insect fingerprints; lane B, population fingerprint derived from bulked DNA of 15 insects in population OH. S, standard DNA. The markers (-) indicate polymorphic bands scored as in Table 2.

Newbury 1994, Weising et al. 1995, p. 141). We used a S-state system to score reproducible band intensity variation to account for allele concentration differences in the pooled samples (Hadrys et al. 1993, Tinker et al. 1994). Thus, polymorphisms were identified as missing, weak, or strong bands (scored as 0, 1, or 2, respectively). Bands between 300 and 2,000 base pairs were scored. Occasionally, some primers did not generate amplification fragments in the higher molecular weight range with some samples. In those cases, all samples were scored relative to the range of the most limited sample. Genetic diversity, h, for each band position (character) was determined as $h = 1 - (x^2 + y^2 + z^2)$, where x, y, and z equal the frequency of absent (Os), weak (Is), or strong (2s) bands, respectively, and the average genetic diversity of all bands = H (*see* Nei 1987, p. 177).

Data were analyzed with the PAUP 3.1 software (Swofford 1991) from the data set of multistate characters, where character states are related to each other through a linear series of unpolarized, unweighted steps (state 0 <> state 1<> state 2). Parsimony trees were derived by heuristic methods (Swofford 1991). The data were analyzed phenetically with SPlus (STATSCI, Seattle, WA). The distance between 2 populations (d_{ij}) was determined as the mean of the sum of absolute differences given by

$$d_{ij} = \frac{1}{C_{ij}} \sum_{k=1}^{C_{ij}} |x_{ik} - x_{jk}|,$$

where C_{ij} is the number of characters that are scored in both populations, and x is the score 0, 1, or 2 for the kth character. Subsequently, a dendrogram was produced from the distance matrix of all 8 populations ($i,j = 1,2,\ldots, 8$) by use of the unweighted pair-group method with arithmetic mean.

Table 2. A comparison of scoring frequencies derived from RAPD-PCR for individual (n = 9) and bulked DNA samples of *T. piniperda* (population OH) at corresponding polymorphic band positions with primer B8

Band no.	Individual DNA			Bulked DNA[a]
	strong	Weak	Absent	
1	0.11	0.22	0.67	Absent
2	0.11	0.00	0.89	Absent
3	0.67	0.00	0.33	strong
4	0.11	0.00	0.88	Absent
5	0.44	0.33	0.22	Strong
6	0.44	0.56	0.00	Strong
7	0.89	0.00	0.11	strong
8	0.11	0.00	0.89	Absent
9	0.00	0.11	0.89	Absent
10	0.11	0.00	0.89	Absent
11	0.56	0.34	0.11	Strong

See Fig. 2 for corresponding fingerprints.
[a] 15 insects pooled per bulk sample.

Isozymes. Esterase electrotypes (characters) were scored as 1 = present or 0 = absent for each insect; the frequencies of presence for each electrotype per population were obtained. The frequencies (f) were transformed into a 4-state character set as 1 = very low (f = 0.00–0.09), 2 = low (f = 0.10–0.29), 3 = medium (f = 0.30–0.69), and 4 = high (f = 0.70-1.00). This nonparametric treatment of the data was used because minor changes in frequency are de-emphasized by grouping. As in the analysis of the RAPD data, we assumed that the 4-state characters were related to each other through a linear series of unpolarized, unweighted steps (state 1<> state 2 <> state 3 <> state 4). Dendrograms were generated by PAUP software (Swofford 1991).

Results

Comparison of Fingerprints from Individuals and Bulked Samples. Fingerprints of individual insects and bulked insects from population OH were obtained under identical conditions with primer B8, and were scored for band frequencies, and the average genetic diversity (H) was determined from the polymorphisms generated by this primer (Fig. 2; Table 2). The results were H = 0.265 for the individual samples and H = 0.142 for the bulked samples. The lower genetic diversity of the bulked samples for this primer quantitatively supports a visual impression that the bulking technique produces a simplified primer fingerprint, and supports the earlier report of Michelmore et al. (1991) that the bulking process eliminates low frequency characters in the population sample.

Variability Among Populations. Out of 24 primers screened, 19 replicated satisfactorily and were scored for 112 polymorphic bands (charac-

Table 3. RAPD polymorphic products from different primers in bulked DNA from 8 *T. piniperda* populations in North America

Primer	Base composition	No. bands scored
B4	GGACTGCAGT	3
B6	TGCTCTGCCC	2
B7	GGTGACGCAG	2
B8	GTCCACACGG	7
B11	GTAGACCCGT	8
B17	AGGGAACGAG	10
B19	ACCCCCGAAG	8
M2	ACAACGCCTC	4
MI3	GGTGGTCAAG	7
MI7	TCAGTCCGGG	5
MI8	CACCATCCGT	7
M20	AGGTCTTGGG	4
S1	CTACTGCGCT	7
s2	CCTCTGACTG	12
s3	CAGAGGTCCC	4
s4	CACCCCCTTG	10
S5	TTTGGGGCCT	5
S6	GATACCTCGG	5
s7	TCCGATGCTG	2
		Total 112

ters) with 3 character states (absent, weak, or strong bands) (Table 3). The average genetic diversity (H) of polymorphic characters in all populations was 0.38.

When tallied, the numbers of unique bands (not found in any other population) for each population were IL = 21, IN1 = 7, MI1 = 6, IN2 = 7, MI2 = 2, OH = 4, NY1 = 2, and NY2 = 1. Population IL is outstanding because it has many more unique characters than any other population. In contrast, there were 23 conserved bands (found in all populations) in the data set.

A mean distance matrix of all populations is shown in Table 4. Because polymorphisms were scored as 0, 1, or 2, mean distances could range from 0 (complete identity) to 2 (complete dissimilarity). Population IL had the greatest mean distances from all other populations (range, 0.714-0.991) and an average of means of 0.886 (20.093). Excluding population IL, distances between all other populations ranged from 0.330 to 0.730, with an average of means of 0.594(±0.082) (Table 4). When we constructed a dendrogram by the unweighted pair group method with arithmetic mean from the distance matrix, the resultant tree showed population IL as a clear outlier from a cluster containing all other populations (Fig. 3A).

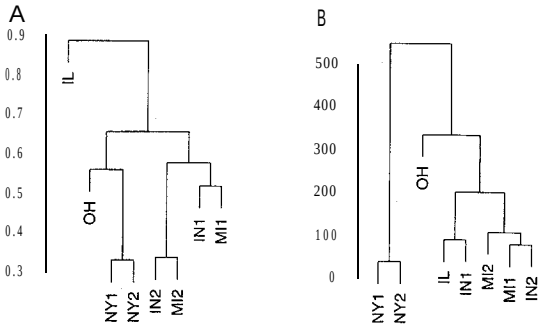

Fig. 3. Dendrograms showing relatedness of the Great Lakes *T. piniperda* populations based on genetic distances (A) or geographical distances in kilometers (B) produced by the unweighted pair group method with arithmetical means.

Dendrograms generated by maximum parsimony method depict possible evolutionary relatedness between the Great Lakes *T. piniperda* populations. The analysis gave 3 minimum and near-minimum length trees (Fig. 4). Tree A has 194 character changes and trees B and C have 193 character changes each. The branch lengths shown are proportional to the number of character changes from the node. In all trees, population IL is isolated from the common ancestral node of the other populations. Furthermore, the IL population shows significant character change from the more distant ancestral node that unifies all populations. One of the trees shows the same general relationships of populations as derived by the unweighted pair-group method with arithmetic mean (compare Fig. 4A with Fig. 3A). In the other 2 trees, the IN1 population (the nearest geographical neighbor to population IL) no longer is contained within the clade of the remaining populations (Fig. 4 B and C).

Parsimony analysis of the isozyme data produced 3 minimum-length trees, all with population IL as an outlier and IN1 in a variable position between IL and other populations. Fig. 4D shows one of the minimum-length trees. Although the isozyme data represents a much more limited survey of polymorphic loci than the RAPD data, the position of population IL as an outlier is still supported.

We also produced a geographically based tree by the unweighted pair group method with arith-

Table 4. Pairwise genetic distances (upper) and geographic distances (kilometers; lower) between 8 *T. piniperda* populations

	IL	IN1	MI1	IN2	MI2	OH	NY1	NY2
IL	—	0.714	0.893	0.893	0.891	0.892	0.991	0.992
IN1	90		0.518	0.589	0.609	0.586	0.634	0.679
MI1	211	124		0.589	0.518	0.640	0.670	0.661
IN2	215	132	80	—	0.336	0.730	0.688	0.696
MI2	309	222	97	119		0.661	0.609	0.618
OH	471	392	305	264	241		0.550	0.568
NY1	693	660	543	535	445	330		0.330
NY2	711	694	571	569	481	358	38	

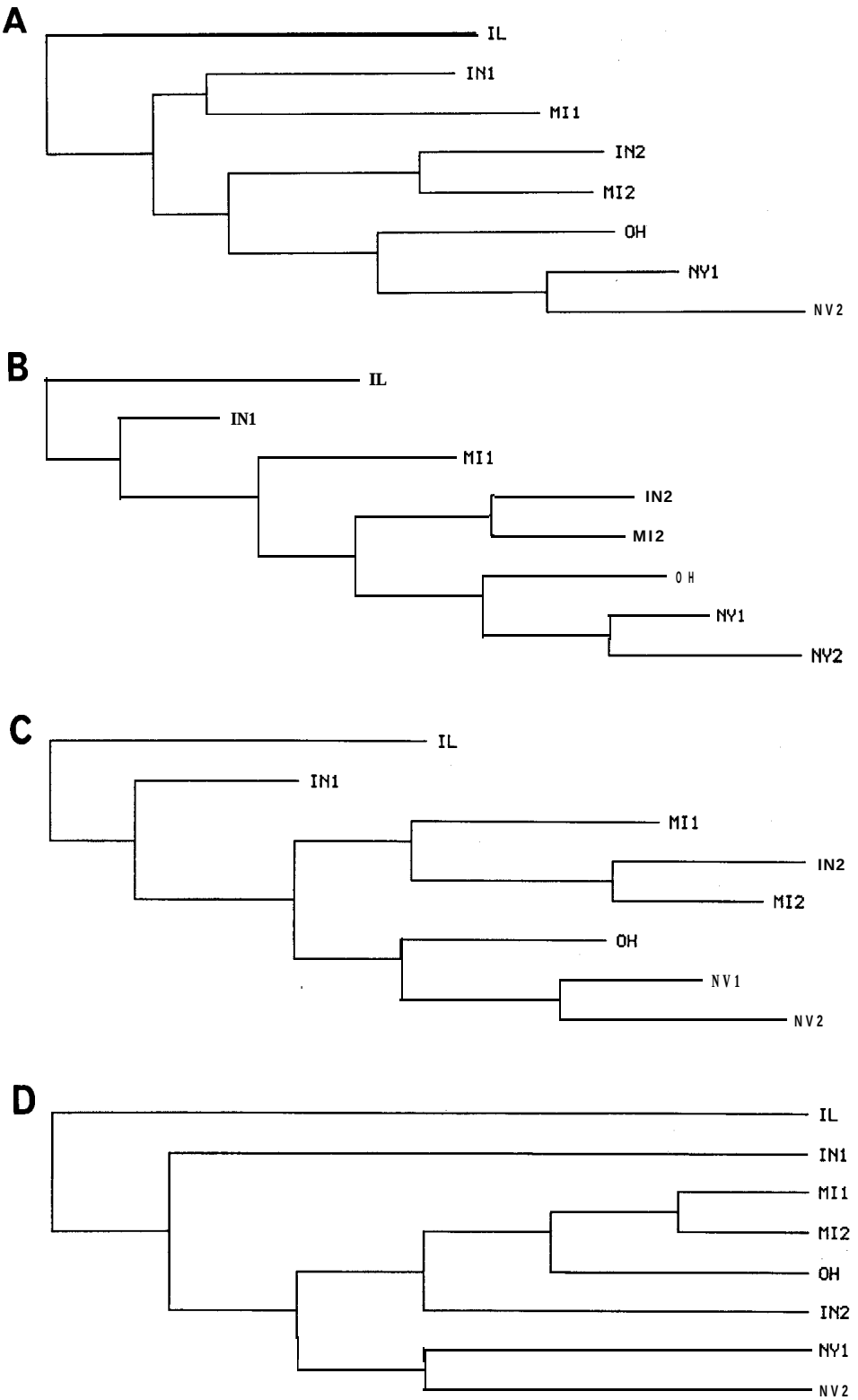

Fig. 4. Dendrograms depicting possible evolutionary relatedness of *T.* piniperda populations derived from maximum parsimony analysis of RAPD data (A, B, and C), and isozyme data (D). In A-C, branch length is proportional to the number of character changes from the node.

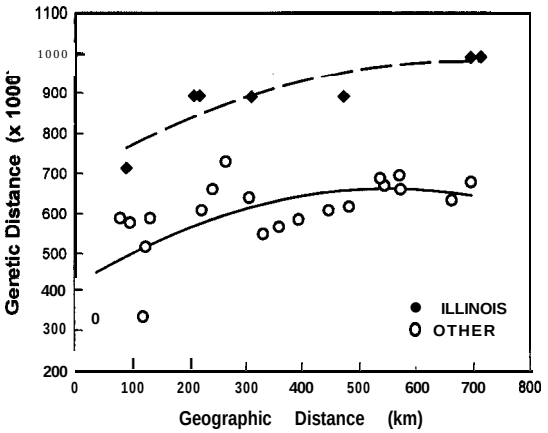

Fig. 5. Relationships between genetic distance and geographic distance for 8 North American *T. piniperda* populations. u, Data points for the 7 relationships in which IL was one of the pair. m, Data points for those relationships in which IL was not included.

metic mean to show relatedness to distance (Fig. 3B). The position of population IL in this geographically based tree showed little relationship to its position in the genetically based trees. In contrast, some of the other populations had similar positions in both the geographically based tree and the genetically based tree.

When the matrix coordinates of Table 4 were graphed, results showed a positive relationship between genetic and geographic distance (Fig. 5). The coordinates of IL x IN1, MI1, IN2, M12, OH, NY1, and NY2 are indicated by ♦ symbols (respectively, from left to right), where $r^2 = 0.78$ (regression analysis, $P < 0.05$). All other coordinates are represented by open circles ($r^2 = 0.46$, $P < 0.05$). Fig. 5 illustrates that IL-related coordinates are separate from all other coordinates based on genetic distance; separation is independent of geographic distance.

Discussion

We used RAPD markers to fingerprint populations of *T. piniperda* with a population bulking strategy. This approach is a powerful means to study genetic variability of natural, outcrossing populations. With our methods, we obtained a consensus fingerprint of a population for each randomly primed PCR reaction. Because we used bulking, uninformative data from individual-specific variation was minimized and polymorphisms representing ≈20% or more of the population were available for scoring (Michelmore et al. 1991). The method was more efficient in time and resources than if we had designed the study by using DNA from 1 individual per reaction. For example, to screen and replicate 15 individuals from 8 populations with 19 primers requires 4,560 PCR reactions; whereas, use of population bulking requires only 304 PCR reactions. By using this method, primers can be efficiently surveyed for informative characters. More primers and more informative characters allow greater confidence that estimates of genetic relatedness are broadly based. Despite reduction in variability caused by bulking, the average genetic diversity of the *T. piniperda* populations was relatively high (0.38), indicating that the bulking strategy, with multistate scoring, is sufficiently robust for characterization of closely related populations.

The dendrograms produced from the RAPD data by parsimony analysis or by the unweighted pair-group method with arithmetic mean show only minor variation in the groupings of the 8 *T. piniperda* populations. The positioning of population IL as an outlier from the other populations is consistent in all genetically based dendrograms (Figs. 3 and 4). Based on the numbers of unique characters associated with each population, the genetic distance data, and the consistency of dendrograms, we conclude that the populations that we sampled were derived from at least 2 founder groups of *T. piniperda* (i.e., founder group 1 for IN1, MI1, IN2, M12, OH, NY1, NY2 and founder group 2 for IL). Because there is a moderate positive relationship between geographical distance and genetic distance among populations (Fig. 5), we suggest that derivatives of founder group 1 dispersed east and west from introductions at 1 or more ports along the southern shores of Lake Erie. Founder group 2, represented in our study only by population IL, may have been founded near the southern tip of Lake Michigan.

An example of population fingerprints that support the separation of *the T. piniperda* populations into 2 founder groups is shown by fingerprints produced by primer B17 (Fig. 6A). In this set of fingerprints, the pattern of population IL is characterized by 2 unique bands (left markers), whereas the other populations show overall similarity including a strong band at 600 base pairs (right marker) that is absent in population IL.

The genetic evidence suggests that insects from the 2 founder groups are interbreeding in the vicinity of population IN1. IL (group 2) was more closely related to neighboring IN1 than to any of the other group 1 populations (Table 4). However, IN1 is clearly a part of group I (Figs. 3A and 6A). Positional variation of IN1 in trees produced by maximum parsimony (Fig. 4 A-C) is consistent with the effects of interbreeding and gene flow between these derivatives of the 2 founder groups.

An illustration of possible interbreeding between insects derived from the 2 founder groups is shown in the consensus fingerprints produced by primer M18 (Fig. 6B). In these fingerprints, where all populations have at least 1 conserved band ("x" in Fig. 6B), neighboring populations IL, IN1, and MI1 are differentiated by a common band not found in the other populations (Fig. 6B, left marker). In this same set of fingerprints, IN1, IN2, MI1,

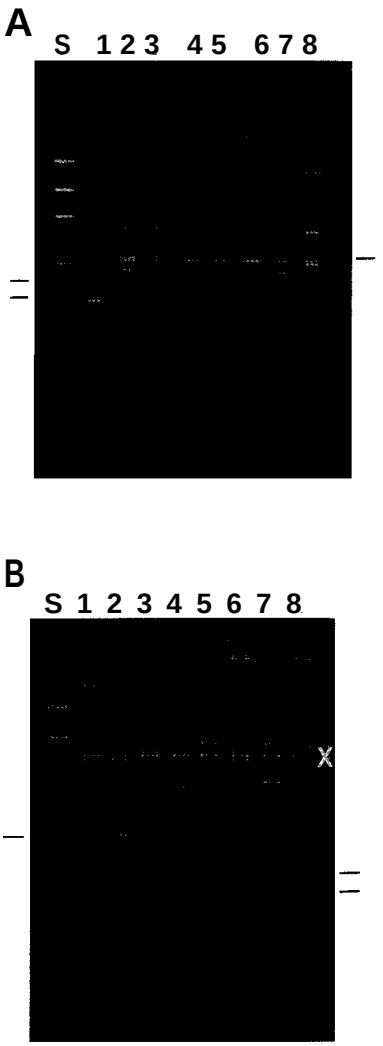

Fig. 6. Population fingerprints of bulked *T. piniperda* DNA from RAPD-PCR using primers B17 (A) and Ml8 (B) showing differences between putative founder groups 1 (lanes 2-8) and 2 (lane 1). Lane 1, IL; lane 2, IN1; lane 3, M11; lane 4, IN2; lane 5, M12; lane 6, OH; lane 7, NY1; lane 8, NY2; lane S, Standard DNA; X, conserved band; –, semiconserved bands.

M12, OH, NY1, and NY2 show their relationship to each other as members of group 1 by similar doublet bands in all populations (right markers, Fig. 6B).

Our study has produced baseline genetic data on North American *T. piniperdu* populations from consensus fingerprints derived from 19 different primers. This new knowledge of North American *T. piniperda* **genetic structure will allow comparisons to be made with indigenous *T. piniperda*** populations worldwide. Particularly relevant is the dis**covery of conserved and semiconserved bands,** some of which are illustrated in Figs. 2 and 6. **These conserved genetic characters may prove** useful as regional markers. Identifying the origin(s) **of the North American founder populations will aid in understanding *T. piniperda* biology and in targeting searches for natural enemies to be used in biological control efforts.**

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