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Molecular and Morphological Analysis of *Dendroctonus pseudotsugae* (Coleoptera: Curculionidae: Scolytinae): An Assessment of the Taxonomic Status of Subspecies

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ABSTRACT *Dendroctonus pseudotsugae* Hopkins infests Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, throughout the distribution of that tree species from British Columbia to northern Mexico. The subspecies *Dendroctonus pseudotsugae barragani* Furniss was described from the mountains of Chihuahua, Sierra Madre Occidental, Mexico, whereas the nominal subspecies, *Dendroctonus pseudotsugae pseudotsugae* Hopkins, occurs north of Mexico. The description of *D. p. barragani* was based on the only known Mexican population at that time. More recently, new populations of this beetle have been discovered at 13 additional localities in Chihuahua, Durango, Coahuila, and Nuevo Leon, Mexico. To test whether these additional populations support the existence of two subspecies, we performed a taxonomic reassessment combining molecular markers (cytochrome oxidase I), morphological characters used in the original description, and newly described morphological characters. Phylogenetic analysis of 89 haplotypes confirms that the Mexican populations are distinct from the sampled populations in the United States and Canada. Morphological analysis indicates that intraspecific variation is greater than previously considered within Mexican populations. However, at least seven characters on the head, pronotum, and elytra (including three previously undescribed characters of frons sculpture) consistently discriminate among Canada-U.S. and Mexico populations. The extension of the known distribution of this beetle in Mexico and verification of its subspecific status will aid in the management and conservation of *Pseudotsuga* in Mexico.

KEY WORDS *Pseudotsuga*, *Dendroctonus pseudotsugae*, mitochondrial DNA, subspecies

Dendroctonus pseudotsugae Hopkins infests Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, throughout the distribution of that tree species from British Columbia to northern Mexico. This bark beetle was first described from Grants Pass, OR (Hopkins 1909). A subspecies, *Dendroctonus pseudotsugae barragani* Furniss, was described from a disjunct population discovered in Chihuahua, Mexico, in 1974 (Furniss 2001). The description of *D. p. barragani* was based on morphological characters of the head, pronotum, and elytra, the manner of oviposition in the parent egg gallery, mating compatibility of beetles from Chihuahua and Idaho, and differences in associated insect fauna (Furniss and Cibrian-Tovar 1980). A recent study on genetic differentiation of *D. pseudotsugae*, using mitochondrial DNA sequences and nuclear DNA markers, showed that genetic differences were geographically correlated, and both markers also revealed that the genetic structure of *D. pseudotsugae* is

influenced by geographic isolation (Ruiz et al. 2009). Observed genetic differences between northern (Canada-U.S.) and southern (Mexico) populations were also consistent within the two groups of populations that likely correspond to subspecies designation by Furniss (2001).

Our goal is to test whether molecular markers and morphological characters allow recognition of monophyletic groups that correspond to at least two different sets of geographically distinct populations (Canada-U.S. and Mexico) in *D. pseudotsugae*. For this study, we acquired adult specimens from 13 new localities in four northern Mexico states (Chihuahua, Durango, Coahuila, and Nuevo Leon) and compared them with specimens from 14 localities in the western United States and British Columbia.

Methods and Materials

Live adult beetles were collected from the bark of infested trees, preserved in absolute ethanol, and stored at -80°C until analysis, or pinned for study and saved as vouchers in the Escuela Nacional de Ciencias Biológicas-IPN (ENCB-IPN) entomological collection. Some DNA samples were provided from colleagues without corresponding voucher specimens

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and were used only in the molecular data analysis. Similarly, specimens loaned from museums were used only for morphological analyses. Samples from 18 populations were used for both analyses. A total of 235 specimens from 24 localities were analyzed for molecular genetic markers, whereas 98 specimens from 22 localities were compared morphologically (Table 1).

Mitochondrial Cytochrome Oxidase I (COI) Amplification and Sequencing. Total genomic DNA was extracted and purified using DNeasy tissue kit (QIAGEN GmbH, Hilden, Germany). Polymerase chain reaction (PCR) amplification of a 600-bp fragment of the mitochondrial cytochrome oxidase I (COI) gene was carried out using primers C1-J-2441 and T12-N-3014 (Simon et al. 1994). DNA amplification was performed using a Biometra T Gradient thermocycler (Biometra GmbH, Hilden, Germany). Samples were preheated for 5 min at 94°C, followed by 35 amplification cycles of 30 s at 94°C, 1 min at 51°C, 2 min at 72°C, and a final extension of 5 min at 72°C. Each PCR reaction mixture contained 50 ng of DNA, 1 μ l of primer (50 pM), 4 μ l of dNTPs (10 mM) (1 μ l for each one), 6 μ l of MgCl₂ (25 mM), 5 μ l of 1 X buffer, and 0.4 μ l of Taq DNA polymerase. The final volume was brought to 25 μ l with ultrapure water. All PCR reactions included negative controls to detect possible contamination.

PCR products were purified using GFX PCR DNA and gel band purification kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom). Cycle sequencing reactions were performed with Big-Dye fluorescent chemistry reaction (Applied Biosystems, Foster City, CA). Both DNA strands were sequenced in an ABI 377 sequencer, and editing of nucleotide sequences and manual alignment were performed using the software program SEQUENCHER, version 4.0.5 (Applied Biosystems). Sequences obtained correspond to positions 2428 and 2977 in the *Drosophila yakuba* Burla mitochondrial genome (accession no. X03240), and are available in GenBank under accessions EU043405-EU043464 and EU193124-EU193152. The compiled DNA data matrix consists of 89 haplotypes of 550 bp.

Sequence Analysis. Phylogenies were constructed using parsimony optimality criterion, with PAUP*, version 4.0b10 (Swofford 2002) and MacClade, version 4.08 (Madison and Maddison 2003). The COI sequence of the sibling species *Detidrocionus simplex* LeConte was included as outgroup (accession AF067985). Cladograms were generated using heuristic tree searches starting with 100 random stepwise addition replicates with the tree bisection and reconnection (TBR) option. All other settings were default. Nonparametric bootstrap values were established with 1,000 replicates and default PAUP* settings. Support for individual nodes on the resulting strict consensus cladogram was determined by calculation of Partitioned Bremer Support values (Bremer 1994), by using TREEROT, version 2 (Sorenson 1999) in conjunction with PAUP*.

We also performed a maximum likelihood analysis, with a heuristic search strategy, ACCTRAN, and TBR

as branch swapping algorithm. To select the best-fit model (s) of nucleotide substitution that best describe the data, we used both the Akaike Information Criterion (AIC) and hierarchical likelihood ratio test (hLRT), as implemented in Modeltest, version 3.7 (Posada and Crandall 1998). Both of them supported the Tamura-Nei model (Tamura and Nei 1993) (TrN) with gamma (G) distributed rate variation and estimated proportion of invariable sites (I). A likelihood ratio test was performed to determine whether the trees obtained followed a molecular clock. To test for monophyly, we performed the Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999). All analyses were performed using PAUP*. The assessment of pairwise sequence divergence (as well as corresponding mean, standard deviation and range), by using the Jukes-Cantor model of nucleotide substitution (Jukes and Cantor 1969), allowed us to test whether standardized percent nucleotide sequence divergence is useful to diagnose and assess subspecies boundaries (Hebert et al. 2003).

Morphological Analyses. Thirteen external morphological characters of specimens were examined at magnifications up to 90X to evaluate the patterns of variation within the species range and their taxonomic utility. These characters included those by Furniss (2001) and three new characters on the frons (Table 2). A subset of these specimens was photographed with a JEOL JSM-5800LV scanning electron microscope (JEOL, Tokyo, Japan) under an acceleration voltage of 15 kV.

Twelve of these morphological characters were coded for analysis under a phylogenetic framework (Table 2). The characters used by Furniss (2001) were reevaluated to code them in a precise and unambiguous way. For example, the sculpture of punctures on pronotum was coded into four character states (categories) according to the number of punctures per 0.01 mm² on the posterior half of the left side. Likewise, the relative size of crenulations on the base of the elytra was expressed as the ratio of the average width of crenulations 2-6 to the width of elytron base. In addition, the relative size of tubercles on interstriae of elytral declivity in females was coded as the average basal width of five tubercles on the second interstria of the declivity divided by the interstria width, and these ratios were then divided into classes as character states.

Finally, phylogenetic analysis of morphological characters was carried out using a heuristic search strategy, with TBR branch swapping, starting with 1,000 random addition sequences. Nonparametric bootstrap values were established with 1,000 replicates and default PAUP* settings. Analyses were performed with the PAUP* program.

Reconciling Data Sets. To assess character compatibility of both molecular and morphological data, we used an incongruence length differences test (ILD; Farris et al. 1995) as implemented by the partition homogeneity test in PAUP*. The test was used to find whether the matrices formed from the original separate data sets were significantly less homogeneous than those from randomly partitioned from the com-

Table 1. Locations, geographic references, number of specimens analyzed, and accession numbers in *D. pseudotsugae*

Location	Key	Latitude	Longitude	No. samples (morphology)	No. samples (DNA data)	GenBank accession no.
Sierra Madre Oriental (SMOR)						
Santa Rita, Nuevo León	SNLO	25° 09' 12" N	100° 08' 41" W		7	EU043405 , EU043409, EU043406 , EU043408, EU043407
Monterreal, Arteaga, Coahuila	MCOA	25° 14' 32" N	100° 26' 37" W	5	10	EU193124 , EU193125, EU193126, EU193127 , EU193128, EU193129
Los Lirios, Arteaga, Coahuila	LCOA	25° 20' 02" N	100° 33' 51" W	3	8	EU193130 , EU193131, EU193132
Rancho los Angeles, Arteaga, Coahuila	ACOA	25° 26' 14" N	100° 42' 30" W	5	12	EU043410 , EU043415, EU043411 , EU043414, EU043412 , EU043413
El Pilar, Arteaga, Coahuila	PCOA	25° 17' 12" N	100° 30' 31" W	5		
La Ciruela, Arteaga, Coahuila	CCOA	25° 14' 03" N	100° 27' 01" W	5		
Sierra Madre Occidental (SMOC)						
Ejido Cienega de la Vaca, San Dimas, DGO	CDGO	24° 05' 20" N	105° 31' 00" W		9	EU043427 , EU043426 , EU043425 , EU043418
Ejido Puentesillas, San Dimas, DGO	PDGO	24° 21' 10" N	105° 54' 39" W	5	11	EU043423 , EU043421 , EU043424 , EU043422 , EU043418
Ejido La Manga, San Dimas, DGO	MDGO	24° 22' 08" N	105° 58' 15" W	5	9	EU043416 , EU043417 , EU043409 , EU043408 , EU043418
Ejido Nuez, San Dimas, DGO	EDGO	24° 22' 29" N	105° 55' 39" W	5	10	EU043419 , EU043420 , EU043408 , EU043421 , EU043418
Llano Grande, Guanasevi, DGO	LDGO	26° 04' 16" N	106° 17' 15" W	5	9	EU043428 , EU043408 , EU043430 , EU043429 , EU043422
Ejido El Nopal, Guadalupe y Calvo, CHI	ECHI	26° 05' 31" N	107° 02' 11" W	5	9	EU043416 , EU043428 , EU043431 , EU043432 , EU043418
Ejido Catedral, Guadalupe y Calvo, CHI	CCHI	26° 12' 40" N	106° 33' 49" W	2	5	EU193133 , EU193134
San Juanito, Bocoyna, CHI	JCHI	27° 58' 09" N	107° 36' 27" W	5		
Southwestern United States (SWUSA)						
Coronado NF, AZ	CRAZ	32° 50' 54" N	109° 43' 01" W	3	10	EU193135 , EU193136 , EU193137
Apache Sitgreaves NF, AZ	APAZ	34° 24' 29" N	110° 02' 21" W	4	11	EU193138 , EU193139 , EU193140 , EU193141
Flagstaff, Coconino NF, AZ	FLAZ	35° 17' 54" N	111° 42' 54" W		13	EU043436 , EU043438 , EU043434 , EU043435 , EU043437 , EU043439 , EU043433 , EU043440
Peaks, Coconino NF, AZ	PKAZ	35° 21' 30" N	111° 33' 53" W	5	9	EU193142 , EU193143 , EU193144 , EU193145 , EU193146
Culbertson, TX	CBTX	31° 56' 49" N	104° 51' 07" W	3		
Northwestern United States and Canada (NWUSA)						
Pinedale, WY	SBWY	42° 52' 10" N	109° 52' 05" W	5	10	EU193147 , EU193148 , EU193149 , EU193150 , EU193151 , EU193152
Vinegar/Vincent Drainage, Malheur NF, OR	JDOR	44° 34' 53" N	118° 31' 21" W	5	11	EU043445 , EU043446 , EU043444 , EU043447 , EU043448 , EU043449
Balm Creek Reservoir, WWNF, OR	BCOR	44° 58' 49" N	117° 33' 44" W		11	EU043445 , EU043444 , EU043458 , EU043457
Mount Hebo Rd, Siuslaw NF, OR	MHOR	45° 10' 30" N	123° 40' 08" W	5	11	EU043443 , EU043445 , EU043444 , EU043441 , EU043442
Spring Creek, WWNF, La Grande, OR	SCOR	45° 20' 25" N	118° 18' 50" W	5	11	EU043445 , EU043449 , EU043455 , EU043452 , EU043454 , EU043450 , EU043456 , EU043451 , EU043453
Mount Emily Rd, WWNF, La Grande, OR	EROR	45° 25' 29" N	118° 08' 32" W	3	9	EU043462 , EU043445 , EU043463
Drumhill Ridge, WWNF, La Grande, OR	DRHL	45° 27' 53" N	118° 11' 25" W		10	EU043460 , EU043444 , EU043459 , EU043461
Lubrecht Experimental Forest, Missoula, MT	MSMT	46° 53' 10" N	113° 26' 55" W		11	EU043445 , EU043444 , EU043463 , EU043448 , EU043441 , EU043442 , EU043464 , EU043453
Revelstoke, British Columbia	RVBC	51° 08' 15" N	118° 16' 26" W	5	9	EU043445 , EU043444 , EU043441 , EU043442 , EU043464 , EU043453

Haplotypes (accession numbers) found in only one population are shown in bold.

Table 2. Morphological character state distribution of *D. pseudotsugae*, as reported by Furniss (2001) and described in the present study

Feature	State	Furniss (2001)		This study	
		A ^a	B ^a	A ^a	B ^a
1. Color	"Elytra and body uniformly dark brown to black; head always darker except all black specimens"	x		x	x
	"Elytra often reddish"		x	x	x
2. Relative depth of epicranial suture	Deeply impressed	x		x	
	Shallowly impressed		x		x
3. Anterior sutures of prepecta	Joined almost perpendicularly (at an angle close to 90°)	x		x	x
	Joined forming a slight curvature close to their joining point		x	x	x
4. Punctures on pronotum	"More widely spaced, generally more coarse"	x			
	"More closely spaced, generally finer"		x		
	11–12 punctures on pronotum per 0.01 square mm			x	x
	13–14 punctures on pronotum per 0.01 square mm			x	x
	15–16 punctures on pronotum per 0.01 square mm			x	x
	17–18 punctures on pronotum per 0.01 square mm			x	x
5. Median line on pronotum	Anterior one third forming a carina separating a shallow depression on either side	x		x	
	Anterior one third without a carina		x		x
6. Crenulations on basal margins of elytra	"Larger, more uniform in size and shape"	x			
	"Smaller, less uniform in size and shape"		x		
	Ratio of relative size of basal crenulations = 0.060–0.069			x	x
	Ratio of relative size of basal crenulations = 0.070–0.079			x	x
	Ratio of relative size of basal crenulations = 0.080–0.089			x	x
	Ratio of relative size of basal crenulations = 0.090–0.099			x	x
7. Margin of striae punctures on elytra	Distinctly elevated, particularly in the anterior part	x		x	
	Flat, with no elevation (margin flush with integument)		x		x
8. Crenulations on interstriae of elytra	"More coarsely rugose" (i.e., bigger and abruptly elevated crenulations)	x		x	x
	"More finely rugose" (i.e., small and just slightly elevated crenulations)		x	x	x
9. Interstitial tubercles on elytral declivity (in females)	"Larger, more uniform in size"	x			
	"Smaller, more variable in size"		x		
	Ratio of width of tubercles and the second interstriae on elytral declivity = 0.36–0.40				x
	Ratio of width of tubercles and the second interstriae on elytral declivity = 0.41–0.45			x	x
	Ratio of width of tubercles and the second interstriae on elytral declivity = 0.46–0.50			x	x
	Ratio of width of tubercles and the second interstriae on elytral declivity = 0.51–0.55			x	
10. Relative width of interstriae 2 on elytral declivity	Width constant through its whole extension or uniformly, gently tapered	x		x	
	Width distinctly constricted along its extremity, generally near its median portion		x		x
11. Median impression on the frontal region	Present and conspicuous			x	
	Absent (or barely visible, with SEM)				x
12. Distribution of crenulations on central area of frons	Forming concentric series of ridges around the median area of frons			x	
	Absent on the central area at the middle of the frons				x
13. Relative abundance of tubercles on the lower frons region	Present but few in the central area of the frons; slightly more abundant toward the epistomal region			x	
	More abundant, particularly in the lower middle of the frons				x

Diagnostic characters are in bold.

^a A, *D. p. barragani*; B, *D. p. pseudotsugae*.

bined data set. When statistical significance is found, the distribution of phylogenetic information is not homogeneous between these original data matrices, suggesting incongruence between these data.

Results

Molecular Data Analysis. No gaps were found along aligned sequences, and editing of 235 COI sequences resulted in 89 different haplotypes of 550 bp. The average nucleotide composition was A + T rich (A = 0.29, C = 0.16, G = 0.13, T = 0.42), and the most

informative nucleotide sites occupied the third codon positions. The low observed rates of change in first and second positions (nt 1 = 2.2%, nt 2 = 0%, and nt 3 = 14.0%) are in agreement with the predicted model of amino acid conservation among insects (Lunt et al. 1996).

With unweighted parsimony analysis using 550 molecular characters, we found 1,640 equally parsimonious solutions. One of them is shown in Fig. 1. The tree shows a good basal resolution (many clades exhibited relatively high branch support values, including almost all internal branches), especially among two

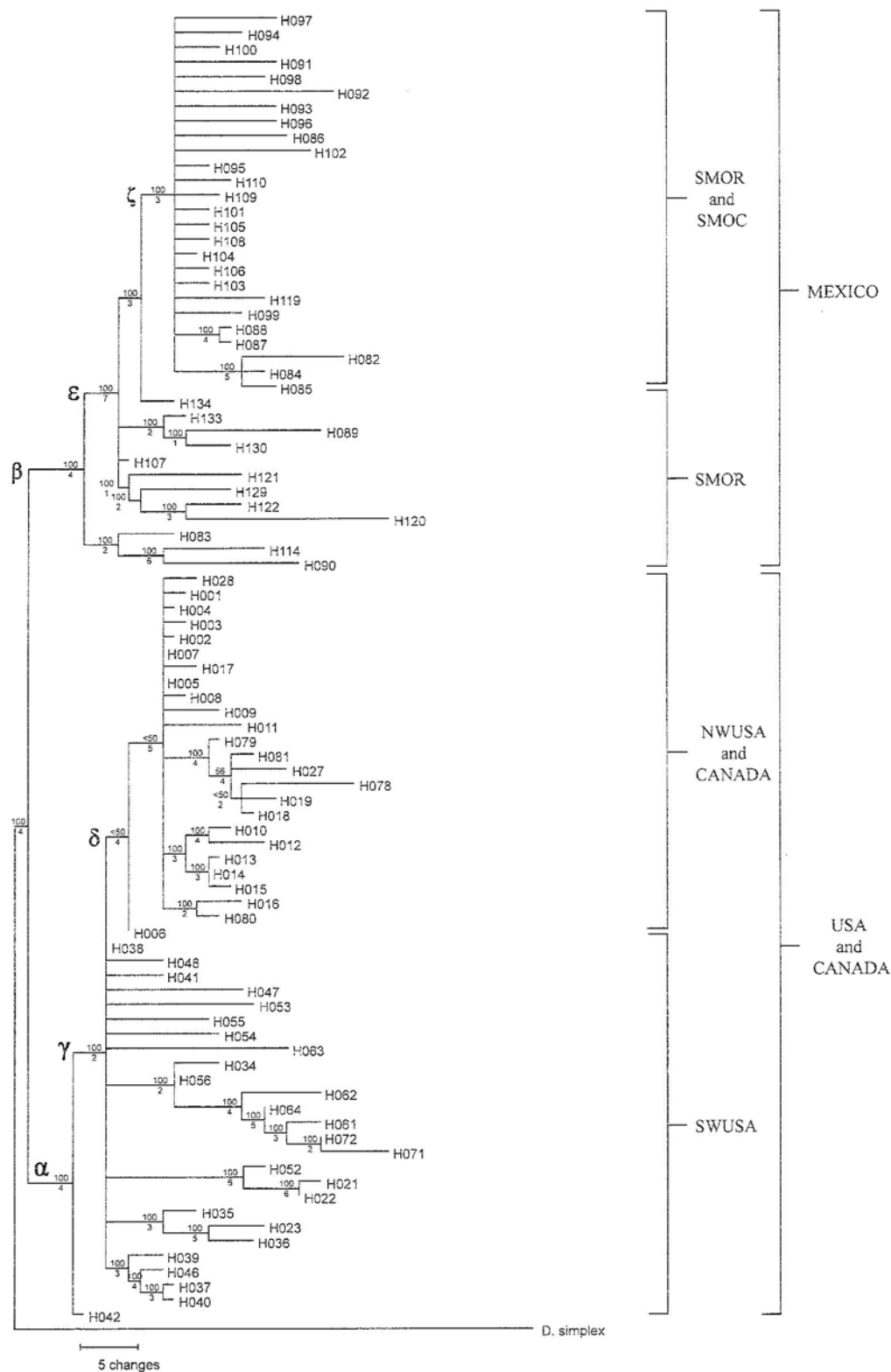


Fig. 1. Phylogram of *D. pseudotsugae* individuals inferred from 550 nucleotides of the mitochondrial COI gene. The tree is one of 1,640 most parsimonious trees (TL = 472, CI = 0.35, RI = 0.73, RC = 0.26) found after a heuristic search using PAUP* (see text for details). Bremer support values (below) and Bootstrap support values (above) are given for each internal branch.

Table 3. Mean percentage of sequence divergence (using the TrN+I+G substitution model) of *D. pseudotsugae* populations by geographic range

Geographic range	Pop	Mean %, SD (range)
NWUSA	SBWY, JDOR, MHOR, SCOR, EROR, RVBC	2.0 ± 1.9 (0.0–10.8)
SWUSA	CRAZ, APAZ, FLAZ, PKAZ	4.4 ± 2.4 (0.4–11.4)
SMOR	MCOA, LCOA, ACOA	4.6 ± 2.4 (1.0–11.4)
SMOC	PDGO, MDGO, EDGO, LDGO, ECHI, CCHI	2.9 ± 2.8 (0.2–16.8)
MEX	MCOA, LCOA, ACOA, PDGO, MDGO, EDGO, LDGO, ECHI, CCHI	4.0 ± 2.7 (0.2–16.9)
CAN-US	CRAZ, APAZ, FLAZ, PKAZ, SBWY, JDOR, MHOR, SCOR, EROR, RVBC	3.9 ± 2.4 (0.0–12.0)
	ALL	6.2 ± 3.9 (0.0–21.2)

well-resolved sister clades which represent monophyletic groups: one that includes haplotypes from Canada and western United States (clade a), and another including only haplotypes from northern Mexico (clade B). It is unclear which one is the basal clade, because both are placed at the same level and are the sister taxon of each other. Within each of these two clades, relationships among haplotypes were less resolved. However, haplotypes from southwestern United States (clade y) are more basal than those of northwestern United States and Canada (clade 8). The haplotypes from the Sierra Madre Oriental are the first from Mexico that arose (clade E), whereas clade S shows little to no nucleotide variation, is poorly resolved, and contains haplotypes from both the Sierra Madre Oriental and Sierra Madre Occidental. Only individuals of Canada-U.S. and Mexico are found in clades a and B, respectively, and they correspond to monophyletic groups. Maximum likelihood analysis resulted in a single tree topology of $-ln = 3129.91.3$ (data not shown). Although less resolved, the tree recovered the same tree topology and two monophyletic groups than maximum parsimony analysis. The Shimodaira-Hasegawa test supports the monophyly of both clades ($P = 0.99$).

The overall intraspecific pairwise mean of sequence differences, calculated with the Jukes-Cantor model (Jukes and Cantor 1969), is 3.9%, almost twice the 2.0% difference frequently used to estimate species boundaries (Avice 2000, Hebert et al. 2003). This result supports previous suggestions that in many groups standardized percent sequence divergence fails to correctly diagnose species boundaries (Cognato 2006). The TrN + I + G model (with $\alpha = 0.533$ for the y-distribution and a proportion of invariable sites of 0.66.5) allowed us to estimate the corrected degree of sequence divergence among all sequences, which ranged from 0 to 21.2%, with an overall intraspecific pairwise mean of $6.2 \pm 3.9\%$ (Table 3). The subdivision in more specific geographic ranges revealed that populations within northwestern United States (NWUSA) have the lowest mean sequence divergence (2.0%), whereas the highest mean (4.6%) is found in the Sierra Madre Oriental (SMOR) (Table

3). An analysis of variance (ANOVA) of these corrected distances showed statistically significant differences among all geographic regions ($P < 0.001$). However, when sequence divergence of northern (Canada and United States) versus southern (Mexico) populations are compared, no statistical difference was found ($P < 0.118$), revealing no difference between these regions. However, when all populations from Mexico (SMOC and SMOR) are compared either with those from southwestern United States (SWUSA) or northwestern United States (NWUSA), high statistical significance is found ($P < 0.001$). A similar result is observed when all northern populations (Canada and United States) are compared with populations either from SMOR or SMOC ($P < 0.001$). Finally, pairwise comparisons of sequence divergence among geographic regions (SMOR, SMOC, SWUSA, and NWUSA) showed high statistical significance ($P < 0.001$) in all cases but one (SMOR and SWUSA, $P < 0.134$). These results suggest that there is a stronger relationship between populations from southwestern United States and the Sierra Madre Oriental than between any other geographic ranges.

Morphological Data Analyses. Our analyses of morphological data confirm the diagnostic validity of some characters proposed in the original description of *D. p. barragani* (Furniss 2001), although other characters proved to be more variable. For example, regarding color, although beetles described by Furniss (2001) were mature and melanistic, beetles collected from other Mexican populations exhibited considerable variability in color. In general, color shows a continuous pattern of variation and varies with maturity of a specimen. Therefore, this character was excluded from the matrix for phylogenetic analysis. In addition to Furniss' characters, we identified three other previously undescribed features (Table 2, characters 11–13) of the frons sculpture as potentially useful for distinguishing among Canada-U.S. and Mexican population groups.

Parsimony analysis of the morphological data set for 98 specimens from 22 populations (plus *D. simplex* as outgroup) yielded $> 10,000$ most parsimonious trees. Topology of the consensus tree (Fig. 8) allowed us to identify two major clades within the ingroup. One of these consists solely of specimens from Canada and the United States, whereas the other includes all specimens from Mexico. These two main clades correspond to a northern (Canada-United States) and a southern (Mexico) group of populations, putatively equivalent to *D. p. pseudotsugae* and *D. p. barragani* sensu Furniss (2001), respectively. Both clades have a high level of support. In contrast, relationships within each one of these clades are poorly resolved. Character optimization for this phylogenetic scenario reveals that seven of the 12 considered morphological characters show unambiguous and consistent differences among Canada-U.S. and Mexico groups of populations (consistency index = 1.00; Table 2). Below, we briefly describe these seven diagnostic characters and their corresponding character states.

Relative Depth of Epicranial Suture. In *D. pseudotsugae* populations from Mexico, the suture located in the upper part of head, in the region of vertex, is deeply impressed, whereas in northern populations this suture is just shallowly impressed and frequently barely visible (Fig. 2).

Median Carina on Pronotum. *D. pseudoisugae* exhibits a median line running longitudinally through the pronotum. Nevertheless, in populations from Mexico, this median line arises as an elevated line forming a "keel" or carina separating a shallow depression on each side of the pronotum, whereas in populations from the United States and Canada this line passes through the pronotum without elevating or forming such a carina (Fig. 3).

Margin of Strial Punctures on Elytra. In northern specimens, punctures forming the striae in the elytra have a flat margin with no elevation; in contrast, in Mexican beetles such punctures show a conspicuously elevated margin, particularly in the anterior part (Fig. 4).

Relative Width of Second Interstriae on Elytral Declivity. In northern populations, the second interstria on elytral declivity maintains its width constant through its whole extension, whereas in Mexican populations interstria 2 has a distinctive constriction along its extremity, generally near its median portion (Fig. 5).

Median Impression on the Frontal Region. A median, transverse, impressed line between the middle of the eyes is present and conspicuous in Mexican beetles, whereas it is apparently absent (or barely visible, under a scanning electron microscope examination) in beetles from Canada and United States (Fig. 6).

Distribution of Crenulations on Central Area of Frons. In Mexican specimens the frontal median line is completely surrounded by crenulate-like granules that form nearly concentric series of ridges around the median area of frons, which also has rather inconspicuous punctures. The sculpture of this area is similar in beetles from Canada and the United States, except that crenulations are absent at the central area of the frons and, therefore, the punctures are more evident (Fig. 6).

Relative Abundance of Tubercles on the Lower Frons Region. Surface of the frons also has tuberculate granules (i.e., knob-like protuberances with rounded tips). In Mexican beetles, these tubercles are present but few in the central area of the frons and become slightly more abundant toward the epistomal region. In beetles from Canada and the United States, these tubercles are clearly more abundant, particularly in the lower middle of the frons, downward to the epistomal region, where they are the most conspicuous element of the sculpture (Fig. 7).

Combined Morphological and Molecular Character Analysis. The ILD test was performed only on samples for which both molecular and morphological data were available (see Reconciling Data Sets). The result of this test did not show sufficient evidence to reject the hypothesis of congruence ($P > 0.01$, $\alpha = 0.05$), suggesting that both kinds of data recover the

same evolutionary history. However, the combined data did not result in a better resolved topology with stronger nodal support than any of the two topologies resulting from the analysis of individual data sets (data not shown).

Discussion

Molecular Character Analysis. Mitochondrial DNA (mtDNA) phylogenetic analysis supports that *D. pseudotsugae* subspecies are discrete entities (i.e., mtDNA lineages), each of them corresponding to a monophyletic group (Fig. 1): one containing only haplotypes from the United States and Canada (clade a) and the other containing only haplotypes from Mexico (clade B). Within these monophyletic groups, potential paraphyly was not observed. Despite low statistical support, however, all populations from northwestern United States and Canada form a clade 8 that clearly arises from a basal clade consisting of southwestern United States haplotypes (Arizona), except Wyoming (clade y), suggesting that northwestern North America was colonized in a general south-north direction with Wyoming possibly having been colonized later. Many haplotypes from Sierra Madre Oriental (SMOR, clade 8) are basal with respect to all those of Sierra Madre Occidental (SMOC, clade ?), suggesting that the latter mountain system was colonized later. However, given the basal position of both a and B clades, the origin of both groups of populations seems to be related to a very specific past event, such as habitat fragmentation of the host. Nonoverlapping intra- and interspecific sequence divergence has been used to establish a standardized percent nucleotide sequence divergence for diagnosing and assessing species boundaries (Hebert et al. 2003). However, this practice has been questioned as sequence divergence (including nuclear and mitochondrial loci) has been found to vary and overlap widely among insects (Cognato 2006).

The observed pattern of sequence divergence is lower within northwestern populations than in southwestern populations (Table 3). This may be the result of several interrelated factors: retreat of glaciers in northwestern North America during Pleistocene warming, with subsequent host recolonization; fragmentation of the occurrence of Douglas-fir in their southernmost distribution; and regional differentiation and local demographic density of populations of *D. pseudotsugae*. At their peak (18,000–24,000 BP), glaciers covered most of British Columbia, and permafrost extended to the Columbia River Gorge, the Cascade Mountain Range in Oregon, and the northern Rocky Mountains (Hewitt and Ibrahim 2001). After this period, many conifers, including Douglas-fir, recolonized the area. Previously, Douglas-fir was distributed throughout most of its current range and considerably farther northward (Hermann 1985). Lower genetic diversity of present day northwestern *D. p. pseudotsugae* may be due to their recent colonization of the area, compared with the more stable southern populations. However, the percentage of

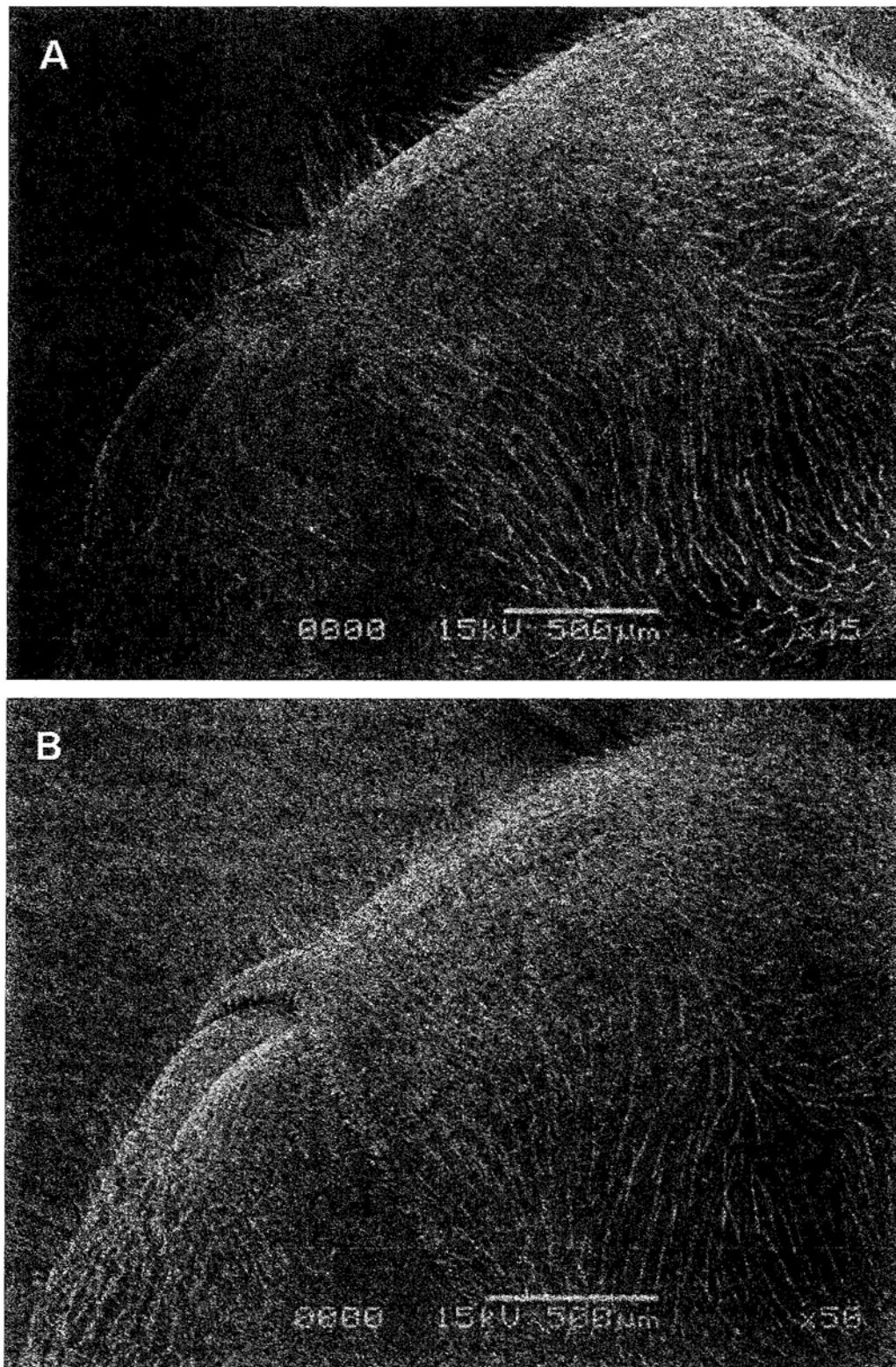


Fig. 2. Relative depth of epicranial suture. (A) Shallowly impressed. (B) Deeply impressed.

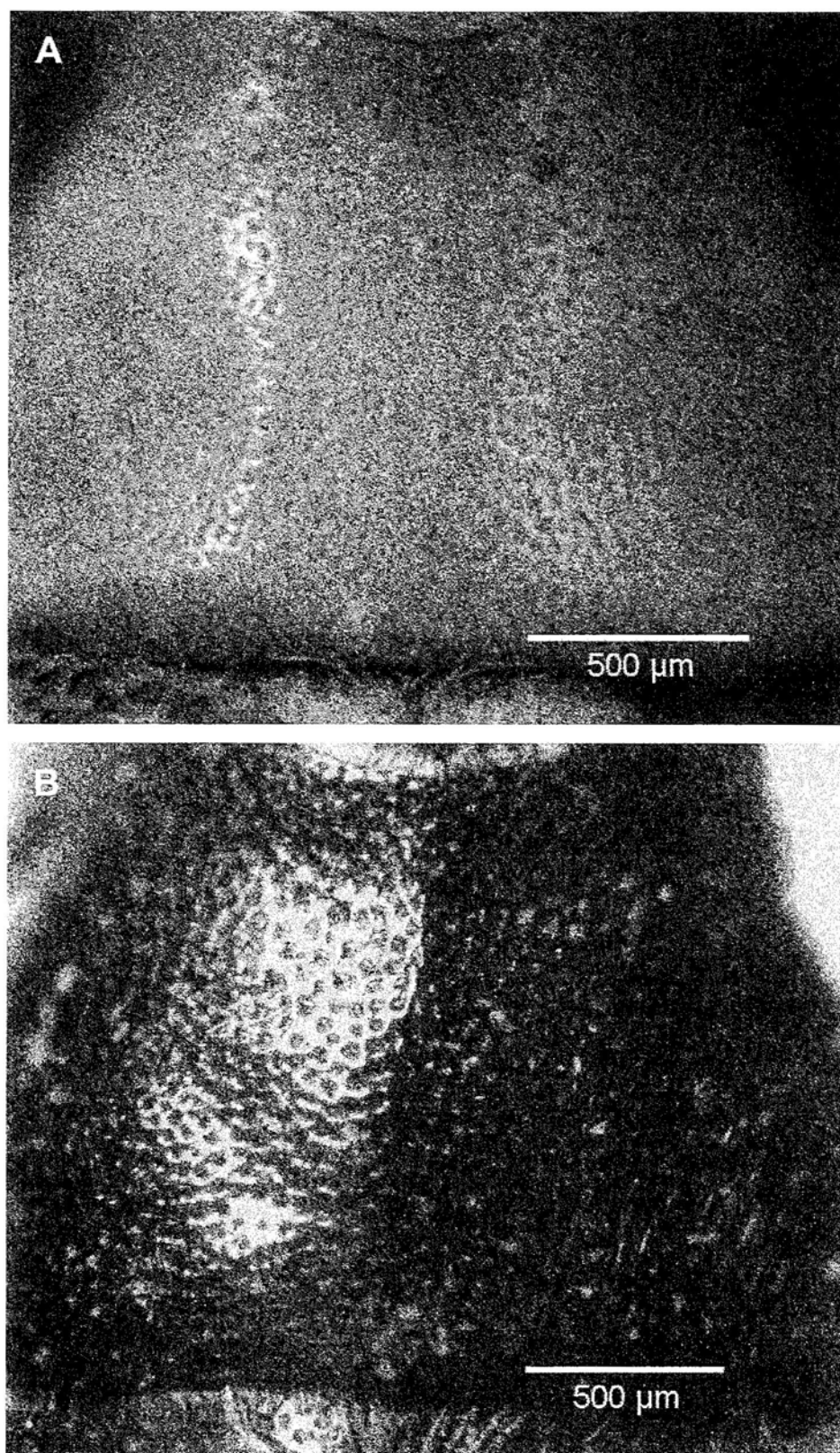


Fig. 3. Median carina on pronotum. (A) Without elevation or carina. (B) Forming a carina on its anterior third.

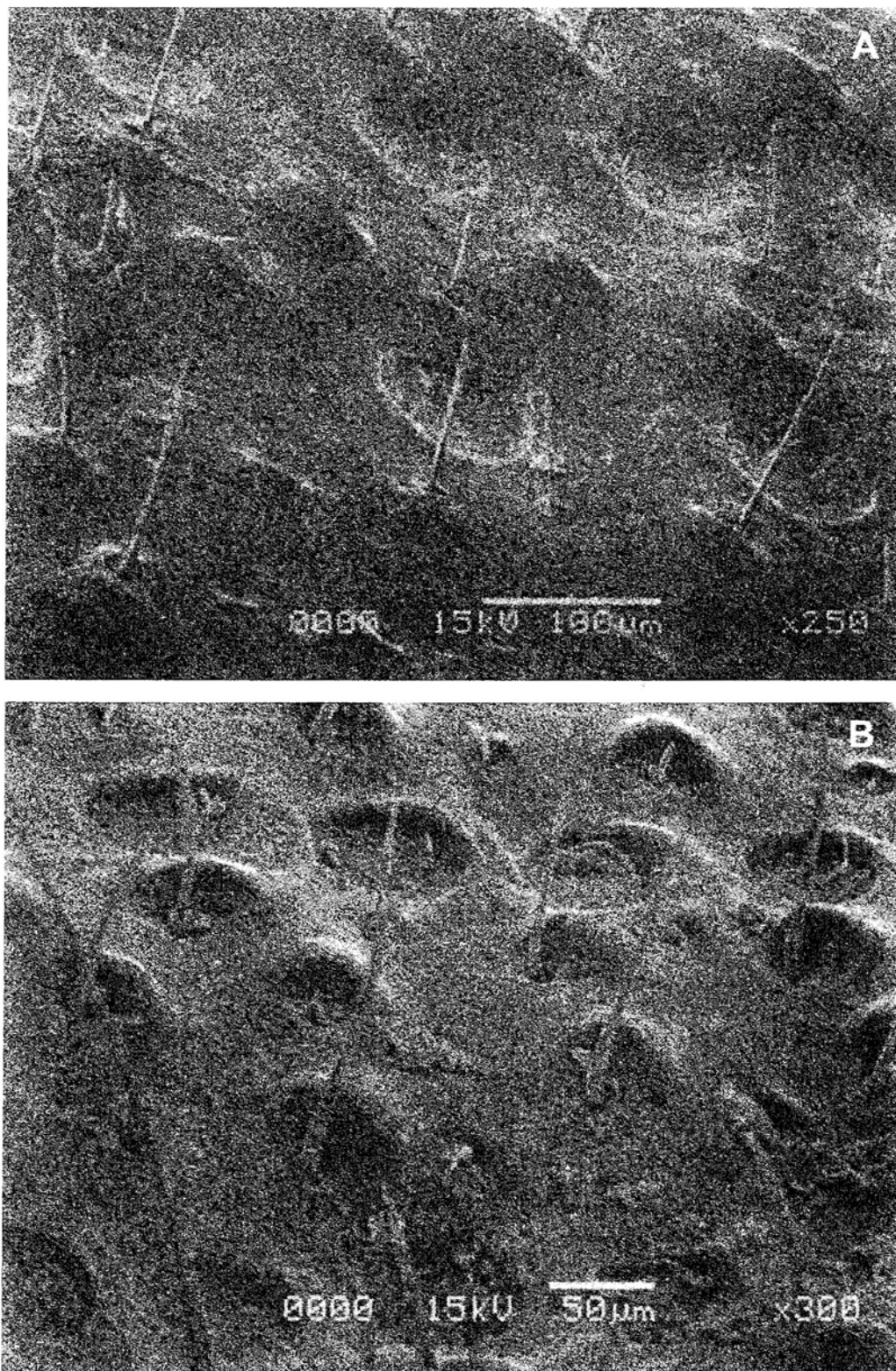


Fig. 4. Margin of striae punctures on elytra. (A) Strial punctures with flat margin. (B) Strial punctures with elevated margin.

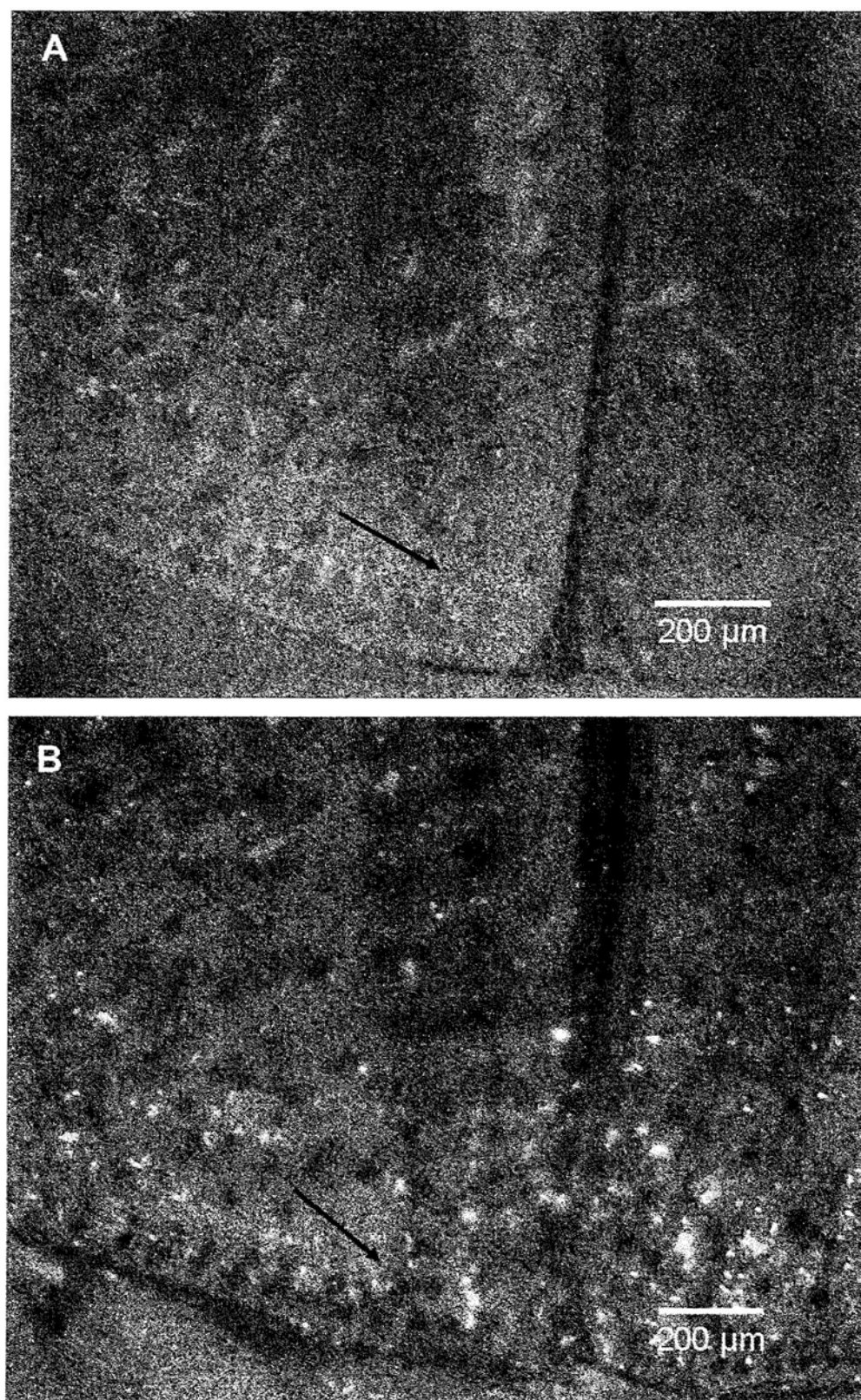


Fig. 5. Interstria 2 on elytral declivity. (A) Constricted. (B) Uniform in width.

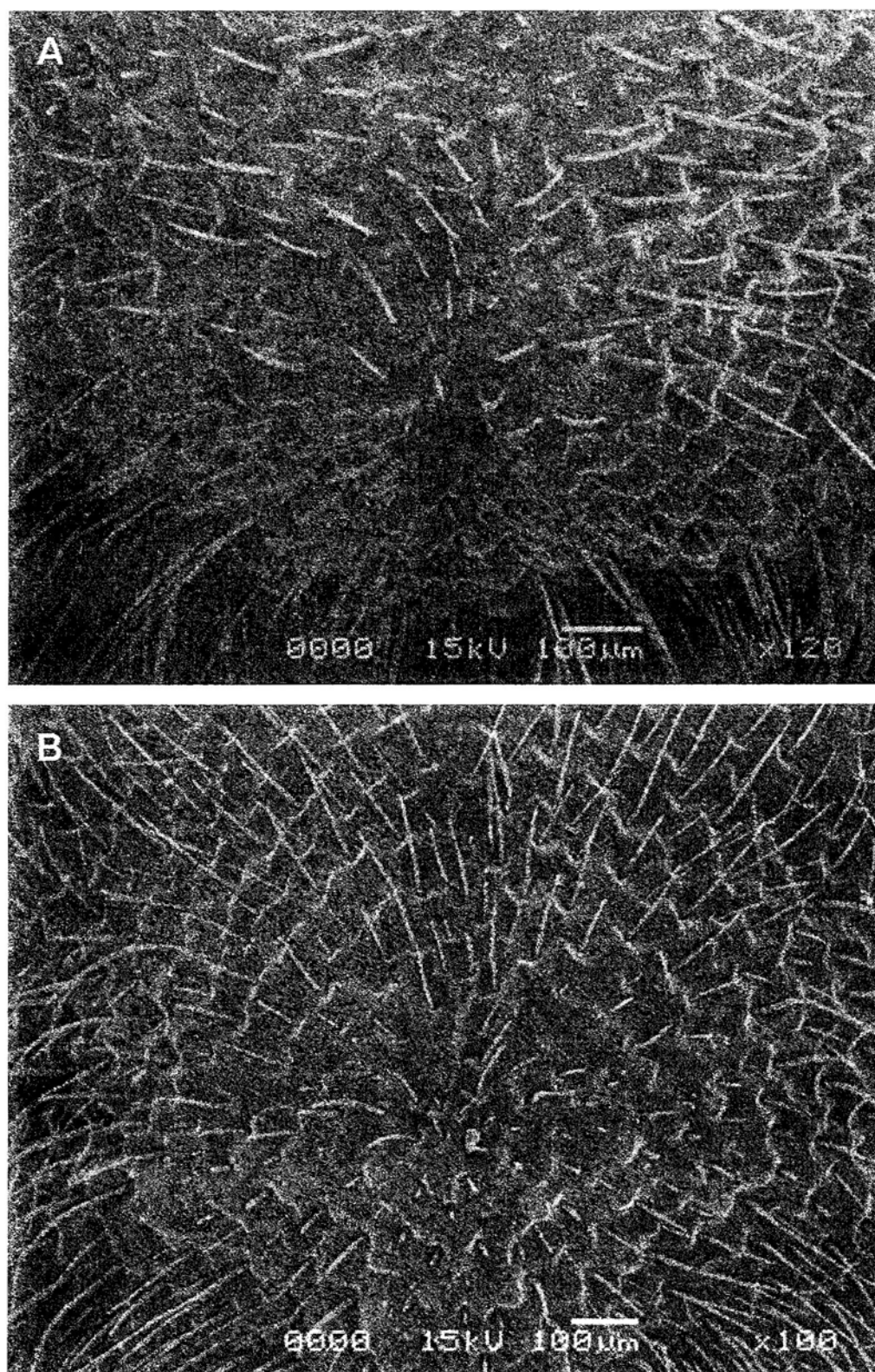


Fig. 6. Frontal region of head. (A) Without a median impression, and without crenulations on the central area. (B) With a well defined median impression, surrounded by crenulations on whole median area of frons.

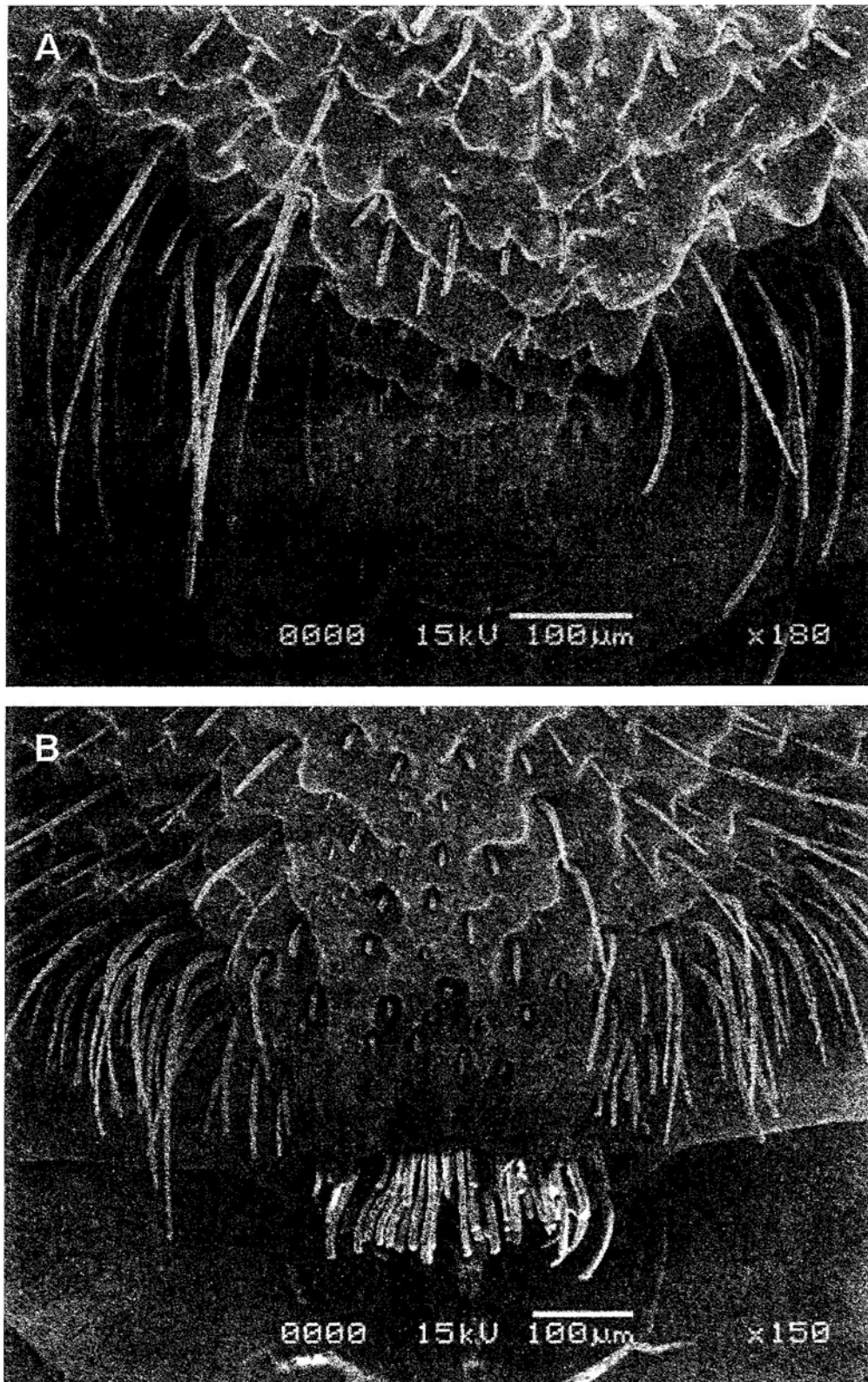


Fig. 7. Tubercles on lower frons region. (A) Abundant, particularly toward epistoma. (B) Scarce, and not covering most of epistoma.

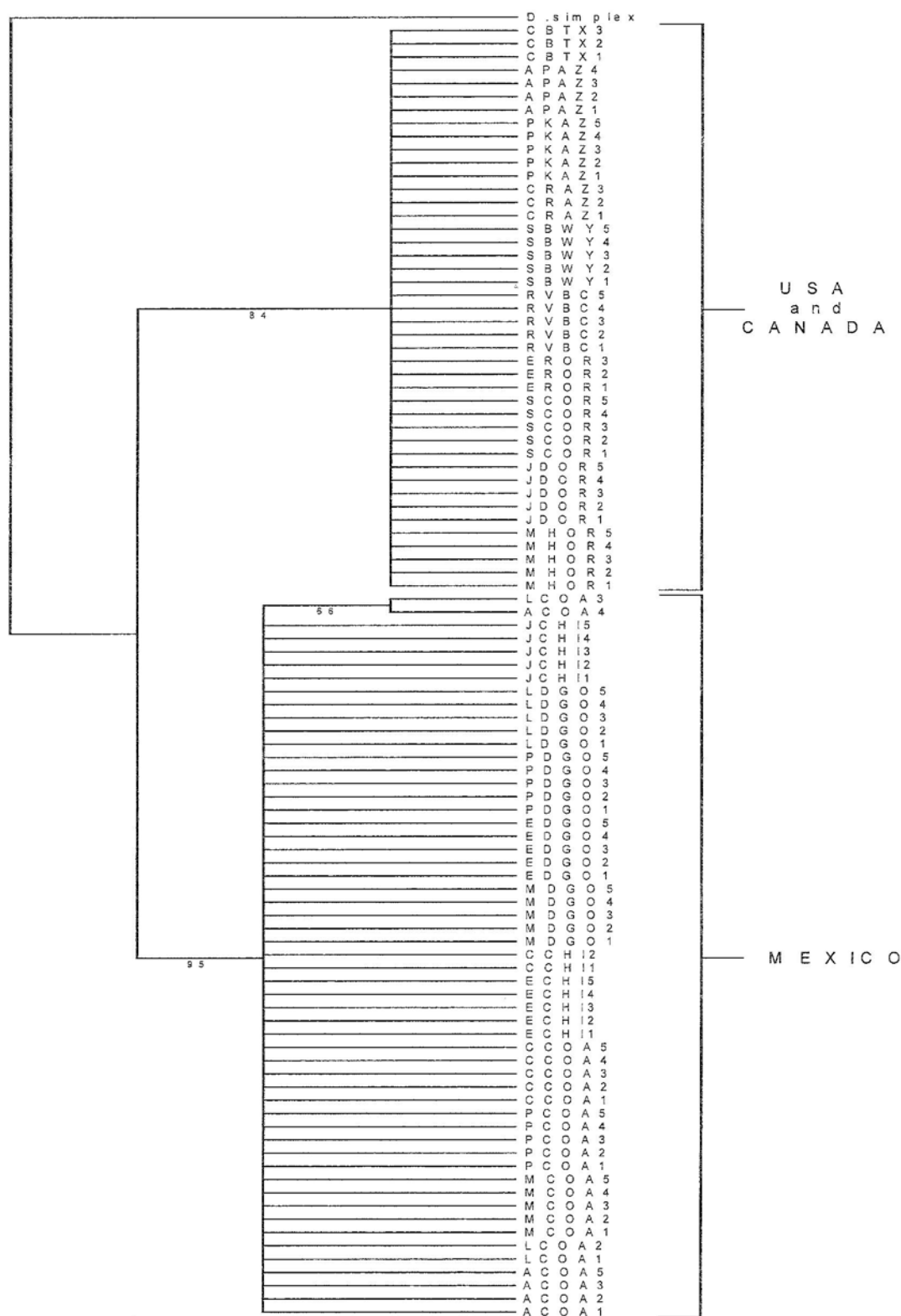


Fig. 8. Cladogram of *D. pseudotsugae* individuals inferred from 12 morphological characters. The tree is a consensus of >10,000 most parsimonious trees (TL = 40, CI = 0.45, RI = 0.95, RC = 0.43) found after a heuristic search. Bootstrap support values (above) are given for each internal branch.

sequence divergence alone is not appropriate for directly testing any of these possible explanations. An examination of likely causes of divergence would require additional data and analyses, such as methods of statistical phylogeography and nested clade phylogeographic, which are beyond the scope of the objectives of this study.

Morphological Character Analyses. Analysis of morphological characters of beetles from the 13 new Mexican localities shows that intraspecific variation within Mexican populations is greater than previously considered (Furniss 2001), particularly due to some differentiation of Coahuila populations with respect to those from Durango and Chihuahua. In addition, Texas specimens (the southernmost location of U.S. populations), are more related to beetles from Canada-U.S. populations than to Mexican populations. Of the 10 morphological characters originally proposed to identify *D. pseudotsugae* subspecies, four show unambiguous and consistent differences among all Mexican and northern populations. The remaining characters identified in the original description of subspecies tend to show overlapping variation among groups of populations, and therefore are taxonomically more labile. However, we found three new features of frons sculpture that also enable separation of northern and southern populations. This combined set of previously described characters plus those proposed in the current study show a consistent pattern of variation among groups of populations found by the phylogenetic analysis. These seven features, therefore, represent a set of diagnostic characters that define each of these two main monophyletic groups.

Evolution of *D. pseudotsugae* Subspecies. Estimates of relationships among *D. pseudotsugae* populations revealed a consensus pattern of differentiation. Most striking is that every phylogenetic reconstruction using either molecular or morphological data yielded two well-resolved groups, each containing only individuals from Canada-United States or Mexico. This study enhanced the sampled range in the southern distribution of the species, and our results show that observed differentiation was not an artifact of incomplete sampling, as the inclusion of only one population from Mexico in the original description could have suggested. The molecular divergence of these two groups, and the morphological differences found strongly suggests that both subspecies could be ranked as full species. Other similar studies have suggested the presence of cryptic species in bark and cone pine beetles (DeGroot and Ennis 1992, Kelley et al. 1999, Cognato et al. 2005). However, additional evidence from other sources (chemical ecology, reproductive isolation, and additional molecular and morphological characters) is still necessary to fully determine if the evolutionary process of speciation is ongoing or completed.

Implications for Management and Conservation of *Pseudotsuga* in Mexico. Finally, the results of this study could be relevant to the management and conservation of Douglas-fir stands in Mexico, where Douglas-fir is a threatened species included in the Norma

Oficial Mexicana NOM-059-ECOL-2001 (SEMARNAT, 2002). For example, differences may exist between subspecies in their pheromones and olfactory response as has been shown to exist in geographically distant populations of another wide-spread North American bark beetle, *Ips pini* (Say) (Lanier et al. 1972). Researchers now have a rational basis for investigating such possibilities.

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