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Source: Annals of the Entomological Society of America, 107(1):11-27. 2014.
Published By: Entomological Society of America
URL: http://www.bioone.org/doi/full/10.1603/AN13047

Two Species Within *Dendroctonus frontalis* (Coleoptera: Curculionidae): Evidence From Morphological, Karyological, Molecular, and Crossing Studies

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Ann. Entomol. Soc. Am. 107(1): 11–27 (2014); DOI: http://dx.doi.org/10.1603/AN13047

ABSTRACT *Dendroctonus frontalis* Zimmermann is considered one of the most important economic and ecological forest pests in the United States, Mexico, and Central America. Recently, two apparent morphological variants of this species were discovered occurring syntopically in Central America and southern Mexico. Morphotype A beetles lack a series of fine parallel ridges on the episternal area of the prothorax that are present on morphotype B. The goal of the present work was to clarify the taxonomic status of the morphotypes of the *D. frontalis* species complex. Geometric morphometric analyses of seminal rod and spermatheca shape together with the characterization of 16 attributes of external morphology revealed differences in quantitative and qualitative characters that distinguished adults of the two morphotypes from each other as well as from the closely related species *Dendroctonus vitei* Wood and *Dendroctonus mexicanus* Hopkins. Karyotype analysis of morphotype B revealed a chromosomal formula (5AA + Xyp) distinct from that found in morphotype A previously reported for *D. frontalis* (7AA + Xyp). In the laboratory, forced intermorphotype crosses produced F1 progeny but at lower frequency than intramorphotype pairings, and dissections of spermatheca revealed a lower frequency of insemination at least one type of heterotypic cross. Phylogenetic analysis of the *D. frontalis* species complex based on 786 bp of the cytochrome oxidase I gene indicated that morphotypes B and A are two independent groups with 98% nodal support within *D. frontalis*. These data provide compelling evidence that the two syntopic morphotypes represent two distinct sibling species.

KEY WORDS seminal rod, spermatheca, geometric morphometry, syntopic species, integrative taxonomy

The genus *Dendroctonus* Erichson (Curculionidae: Scolytinae) is widely distributed in North and Central America and Eurasia. They colonize and kill trees of the genera *Larix* Mill., *Picea* A. Dietr., *Pseudotsuga* Carrière, and *Pinus* L. (Wood 1982). *Dendroctonus* is composed of 19 species, one of them with two subspecies, which are ordered into different groups (Wood 1963, 1982; Lanier 1981; Kelley and Farrell 1998; Zúñiga et al. 2002a). The *Dendroctonus frontalis* complex (sensu lato) is composed of the *Pinus*-infesting species *Dendroctonus adjunctus* Blandford, *Dendroctonus approximatus* Dietz, *Dendroctonus brevicomis* LeConte, *Dendroctonus frontalis* Zimmermann, *Dendroctonus mexicanus* Hopkins, and *Dendroctonus vitei* Wood (Lanier et al. 1988). The composition of the *D. frontalis* complex has changed virtually with almost every taxonomic review (Lanier et al. 1988), and species assignation has been based largely on external morphological features (e.g., setal vestiture and sculpturing of the elytral declivity, frons sculpture, shape of epistomal process, and pronotal size and sculpture. Nevertheless, routine identification of the more difficult species in the *D. frontalis* complex (e.g., *D. frontalis*, *D. mexicanus*, and *D. vitei*) cannot be performed with external morphological features alone, because broad intraspecific morphological variation exists, and key external character states can be distinguished only with practice. In contrast, seminal rod shape and chromosome number have been shown to be reliable features for distinguishing these species (Vité et al. 1975; Lanier 1981; Wood 1982; Lanier et al. 1988; Zúñiga et al. 2002a,b). With the exception of *D. vitei*, species in the *D. frontalis* complex have been characterized by karyological studies (Lanier 1981;

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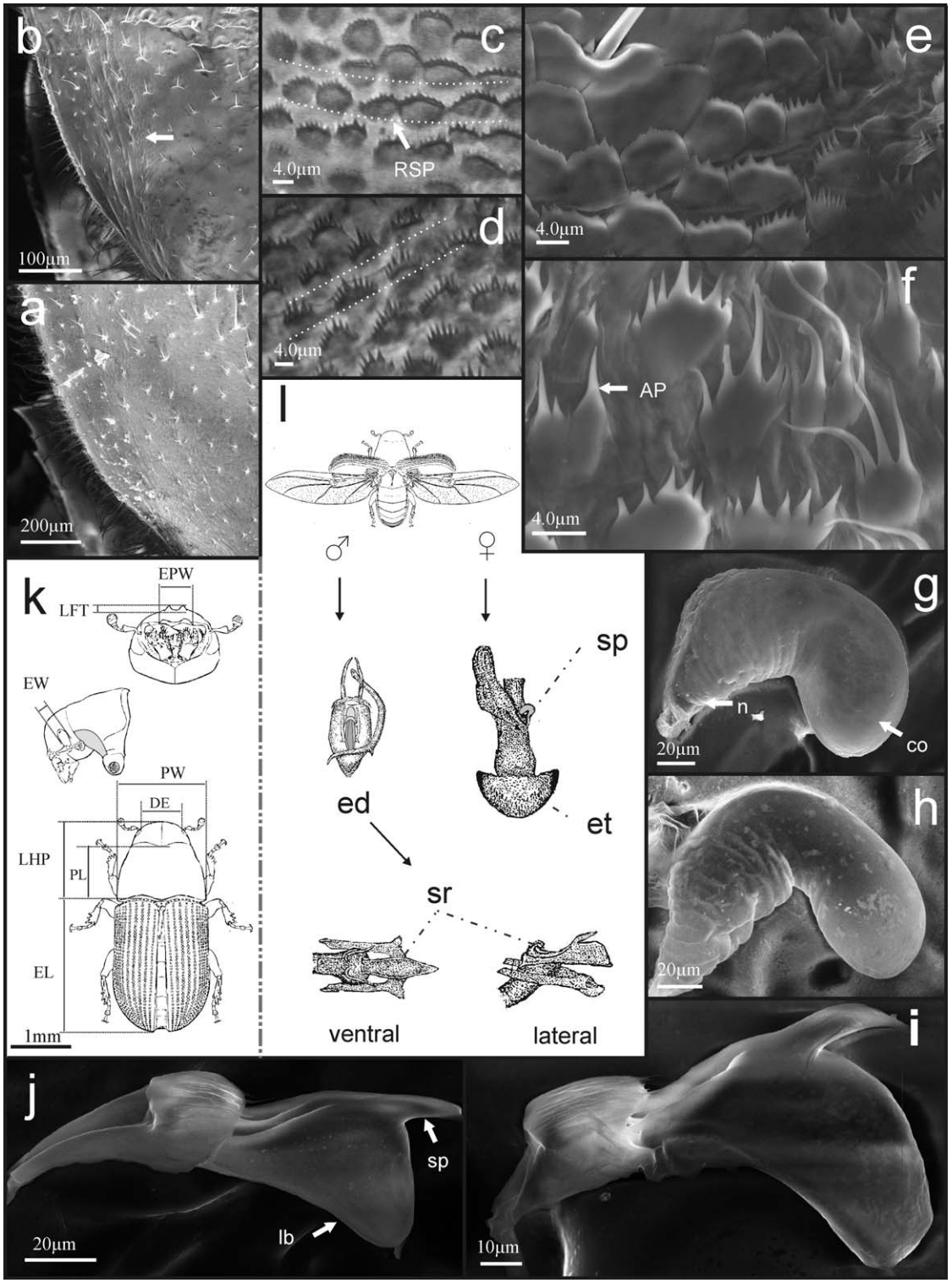

Fig. 1. Analyzed anatomical structures of *D. frontalis* in both MA, morphotype A, and MB, morphotype B. Sculpture of preepisternal area: (a) MA smooth, (b) MB striated; rows of squamiform plates on eighth tergite: (c) MA, (d) MB; acute projections on posterior edge on squamiform plates in the median proximal area of the eighth tergite: (e) MA short and uniform, (f) MB variable in size; spermatheca: (g) MA with round cornu, aggregated striae on the nodulus, and ≈1/4 of body covered by striations, (h) MB with oval cornu, separated striae on the nodulus, and ≈1/2 of body covered by striations; seminal

Lanier et al. 1988; Zúñiga et al. 2002a,b) as well as mtDNA sequences (Kelley and Farrell 1998).

Within the *frontalis* complex, *D. frontalis* (southern pine beetle) is an important economic and ecological forest pest within its extensive geographic range, which includes the southeastern United States, Arizona, Mexico, and Central America (Clarke and Nowak 2009). During periodic outbreaks, *D. frontalis* are capable of overcoming the defenses of healthy pine trees and deforesting patches of the landscape ranging in size from small "spots" to thousands of acres (Billings 2011).

Recently, two morphologically distinct variants of *D. frontalis* were identified in collections from Central America and southern Mexico. Morphotype A (MA) beetles were on average somewhat smaller than morphotype B (MB) beetles, and MA insects of both sexes lacked of a fine series of ridges present in the preepisternal area of the prothorax morphotype MB beetles (Midtgaard and Thunes 2002; Sullivan et al. 2012, Fig. 1; this paper Fig. 1a and b). Although this last character has been used to separate these morphs, a formal analysis has not been conduced to evaluate its utility as taxonomic character. The morphotypes have been found to differ significantly in production of known *Dendroctonus* pheromone components (specimens from Chiapas, Mexico) and in the composition of their cuticular hydrocarbons (specimens from Chiapas and Belize; Sullivan et al. 2012). These data suggest that the *D. frontalis* morphotypes may represented distinct species.

To evaluate the taxonomic status of the two *D. frontalis* morphotypes we analyzed: 1) variation of attributes of external and internal (i.e., genitalia) morphology of both morphotypes; 2) the karyotypes of both morphotypes; 3) the phylogenetic relationship of the two morphotypes with respect to other members of the *frontalis* species complex as determined from mtDNA cytochrome oxidase I sequences; and 4) sperm transfer and reproductive success of laboratory crosses between morphotypes.

Materials and Methods

Samples. In total, 389 *D. frontalis*, 200 *D. mexicanus*, and 22 *D. vitei* from 18, 8, and 2 geographic localities, respectively, were analyzed (Table 1). Specimens were collected by the authors from naturally infested *Pinus* spp. or borrowed from museums. Sex of the specimens was determined by the presence of frontal tubercles and stridulatory apparatus in males (Lyon 1958, Mendoza-Correa and Zúñiga 1991).

Genitalia of males and females were dissected from fresh or alcohol-preserved specimens. The genitalia were cleared by incubating them for 10 min at 10°C in 10% KOH. After incubation, structures were immersed in 20% acetic acid solution to neutralize the KOH and subsequently rinsed with 100% ethanol. The genitalia were then dissected. For females, the eighth tergite and spermatheca from the same individual were mounted on the same slide. For males, the seminal rod was separated from the genital capsule and all parts were mounted on the same slide. All structures were mounted in Hoyer's medium on semipermanent slides for easy manipulation throughout the study.

Character Analysis for the Two Morphs. Based on the presence or absence of striations on the preepisternal area of the prothorax of 389 *D. frontalis* insects (Midtgaard and Thunes 2002; Sullivan et al. 2012, Fig. 1; this paper Fig. 1a and b), we identified 268 specimens striaeless (MA) and 121 with striae (MB). These insects were exhaustively examined to identify additional morphological characters for the correct morphs identification. From 30 reviewed attributes, 16 were taxonomic characters potentially useful for morphotype separation: 10 were on the external cuticle (8 quantitative and 2 qualitative) and the rest were terminalia's characters (in the eighth tergite, spermatheca, and seminal rod).

Adults beetles were examined at magnifications up to 90× and were photographed with an Environmental Scanning Electron Microscope ESEM (Evo 40 VP, Carl Zeiss, Oberkochen, Germany). All body measurements and observations were made using a dissecting microscope with an ocular micrometer (20–60×). Slide-mounted features were observed with a phase contrast microscope (400×).

Character states for each of the following characters were documented for all specimens:

1. Outline of the frons (OF). In lateral view. 1) Truncated convex, 2) completely convex.
2. Number of rows of squamiform plates on the eighth tergite (RSP). The eighth tergite displays parallel rows of squamiform plates. The rows are better defined in the anterior area of the tergite than in the posterior, where they fade (Fig. 1c and d).
3. Acute projections on posterior edge of squamiform plates in the median proximal area of the eighth tergite (AP). 1) Short and uniform (Fig. 1e), 2) variable in size (Fig. 1f).
4. Shape of the cornu of the sphermatheca when viewed laterally (SCS). 1) round, 2) oval (Fig. 1g and h). The spermatheca is reniform and is divided into the nodulus and cornu. The nodulus is the portion between the spermatic duct and the middle constriction of the spermatheca, whereas the cornu includes the distal portion of the spermatheca beyond the middle constriction. The cornu presents variation in length relative to the width of middle

rod: (i) MA with strongly convex posterior margin of lobe, (j) MB with plane posterior margin of lobe; (k) body measures: LFT, length of frontal tubercles; EPW, epistomal process width; DE, distance between eyes; EW, eye width; LHP, length of head-pronotum; PW, width of posterior margin of pronotum; PL, pronotum length; EL, elytral length; (l) female and male genitalia: Ed, aedeagus; sp, spermatheca; sr, seminal rod; et, eighth tergite; RSP, rows of squamiform plates on eighth tergite; AP, acute projections on posterior edges on squamiform plates; n, nodulus; co, cornu; sp, spine; lb, lobe.

Table 1. Populations of two morphotypes of *D. frontalis* and of *D. mexicanus* and *D. vitei* used in analyses

Country	Locality	Coordinates	Host	Number of specimens studied[a]		Type of analysis performed[b]					
				MA	MB	U	M	Sr	Sp	C	G
D. frontalis											
United States											
A	Mississippi, Homochitto National Forest	31.5°, 91.2°	Lindgren trap	10				X			
B	Arizona, Coronado National Forest	31° 48′, 109° 24′	Lindgren trap	6				X			
C	Georgia, Oconee National Forest	33° 47′10″, 83° 14′33″	*P. taeda*	4				X			
Mexico											
D	Querétaro Jálpan, La Parada	21° 06′21″, 99° 05′99″	*P .greggii*	20		X	X	X			
E	Oaxaca, Santa María Albarradas, San Pablo Villa de Mitla	16° 59′33″, 96° 11′15″ E	*P. pringlei*	41	6	X	X	X	X		
F	Oaxaca, San Pedro Ayutla Zacatepec Dto Mixe	17° 07′12,″ 96° 44′56″	*P. pringlei*	21	8	X	X	X	X		
G	Oaxaca Norte	17° 27′44″, 96° 41′38″	*P. pringlei*	50	1	X	X	X	X		
H	Chiapas, La Trinitaria, Parque Nacional Lagos de Montebello	16° 08′42″, 91° 43′29″	*P. oocarpa, P. maximinoi*	70	70	X	X	X	X	X	X
I	Chiapas, Teopisca	16° 31′57″, 92° 28′12″	Lindgren trap	3				X			
J	Chiapas, Motozintla	15° 20′, 92° 15′	Lindgren trap	2		X	X	X			
Guatemala											
K	Sololá, Finca Chuchiya	14° 44′00″, 91° 07′50″	*P. pseudostrobus*	2	10			X			
L	Sololá, Colonia Maria Tecun	14° 48′50″, 91°12′20″	*P. oocarpa*	5	20			X			
M	Sololá, Finca Socorro	14° 45′10″, 91° 08′20″	*P. pseudostrobus*	11	6			X			
N	Huehuetenango, Canselaj	15° 16′10″, 91°25′53″	*P. oocarpa*	4	4			X			
Belize											
O	Mountain Pine Ridge Forest Reserve	16° 59′44.7″88° 46′23″	Lindgren trap	8	9			X			
Honduras											
P	Guaimaca	14° 32′08″, 86° 49′00″	*P. oocarpa*	17	3			X			
Nicaragua											
Q	El Picacho, Cerro Tomabu	13° 02′00″, 86° 18′00″	*P. oocarpa*	10	4			X			
R	Jalapa	13° 55′15″, 86° 07′38″	*P. caribaea*	8				X			
Total				292	141				433		
D. mexicanus											
United States											
S	Arizona, Coronado National Forest		—		3			X			
Mexico											
T	Jalisco, Gómez Farias	19° 52′5″, 103° 24′14″	—		3			X			
U	Distrito Federal,Tlalpan, Totoloapan	19° 18′00″, 99° 12′56″	*P. montezumae*		25		X				
V	Estado de Mèxico, Nicolas Romero	19° 40′32″, 99° 22′43″	*P. montezumae*		39		X	X			
W	Puebla,Tepeyahualco, Hacienda San Roque	19° 29′52″, 97° 30′00″	*P. cembroides*		34		X				
X	Tlaxcala, Altzayoaca, Ejido Santa María Las Cuevas	19° 23′32″, 97° 44′17″	*P. cembroides*		30		X	X			
Y	Veracruz, Perote, Ejido Agua de los Pescados	19° 26′45″, 97° 7′57″	*P. montezumae*		37		X	X			
Z	Chiapas, Las Margaritas, Bajuco	16°27′52″, 91° 53′18″	*P. pseudostrobus*		29		X				
Total											200
D. vitei											
Guatemala											
Aa	Sierra de las Minas Biosphere Reserve	15° 06′05″, 89° 37′20″	*P .oocarpa*		12			X			
Ab	Chimaltenango, Patzun	14° 39′, 90° 58′	—		10		X				
Total											22

[a] MA, morphotype A; MB, morphotype B.

[b] U, univariate analyses of morphological characters; M, multivariate analyses of morphological characters; Sr, geometric morphometric analysis of seminal rod; Sp, geometric morphometric analysis of spermatheca; C, karyotype analysis and crossing experiments; G, molecular phylogenetic analysis.

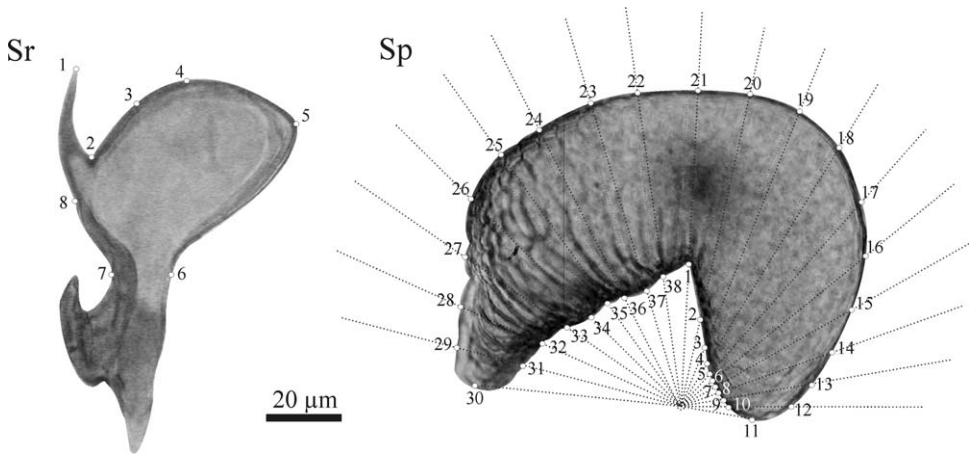

Fig. 2. Sr (seminal rod) and Sp (spermatheca) showing the placement of LM, landmarks, and SL, semilandmarks, for shape analysis: Sr: 1–8 LM; Sp: 11, 30 LM and 1–10, 12–29, and 31–38 SL.

constriction. In the round shape, the length is similar to width of middle constriction; in the oval shape, the length is bigger than width of middle constriction.

5. Number of striae on the spermatheca (NSS). The surface of the spermatheca is ornamented by transverse striae that partially or completely encircle it and are distributed predominantly in the nodulus (Fig. 1g and h). This character was observed in lateral view.
6. Density of striae in the proximal region of nodulus (DS). 1) Aggregate, 2) no-aggregate (Fig. 1g and h).
7. Proportion of the spermatheca covered by striae (PSS). 1) ≈1/4 or (2) 1/2 of spermatheca (Fig. 1g and h).
8. Shape of posterior margin of the seminal rod's lobe in lateral view (SSR). 1) Convex or 2) plane or slightly convex (Fig. 1i and j).

Continuous Characters. We measured the length of the frontal tubercles (LFT), epistomal process width (EPW), distance between the eyes (DE), eye width (EW), length of the head-pronotum (LHP), width of the pronotum at its posterior margin (PW), pronotum length (PL), and elytra length (EL; Fig. 1k).

Data Analyses

Statistical Analyses. Normality of the distribution of each continuous character for each morphotype was tested independently by the Shapiro and Wilkinson (1965) test and examination of distribution histograms. In addition, we tested the homogeneity of variances using Cochran's test (Cochran 1941). Because all characters had heterogeneous variances, all data were standardized $(X_i - X_{mean}/SD)$.

Univariate Analyses. Individual variation between morphotypes was evaluated by Student's *t*-test for continuous characters and the Mann-Whitney test for discrete characters (Zar 2010).

Multivariate Analyses. Each insect was considered an operational taxonomic unit (OTU). Two methods were used: Principal coordinates analysis (PCoA) was used to explore multidimensional patterns of variation among specimens and cluster analysis to evaluate the grouping of specimens by mean of phenograms (Legendre and Legendre 1998). Both methods were performed with a distance matrix computed with the Gower index using 15 characters defined in this study and including the sculpture of the preepisternal area (1] presence or 2] absence of striations on the preepisternal area of the prothorax; PAE) as a character additional. Phenograms were built by the unweighted pair-group method with arithmetic mean (unweighted pair-group method with arithmetic average). Three different comparisons were carried out. In the first two, *D. frontalis* (both morphotypes) males and females were analyzed separately; the third comparison was performed among *D. frontalis* (both morphotypes), *D. mexicanus*, and *D. vitei* of both sexes. The number of characters included in each comparison was different because of the differences in numbers of sex-specific characters examined (see results). Univariate and multivariate analyses and plots were constructed using Matlab 7. 8. 0. 347 for windows (The MathWorks Inc.).

Geometric Morphometrics. We assessed seminal rod and spermatheca shape variation in both *D. frontalis* morphotypes by means of geometric morphometrics analysis. The seminal rods of MA and MB and *D. frontalis* complex species *D. mexicanus* and *D. vitei* from multiple locations were compared (Table 1). Spermatheca analysis was conducted on MA and MB specimens collected in seven localities.

Each seminal rod and spermatheca was photographed in lateral view using a Nikon Coolpix 5000 camera mounted on a phase contrast microscope (400×). Images were identically arranged so that the seminal rod was oriented with the spine (ventral projection) pointing up and the lobe (or dorsal projection) on the right-hand side of the spine; the spermathecae were oriented with the termini pointing downward and the nodulus leftward (Fig. 2).

Landmark Analysis—Seminal Rod. The shape of each seminal rod was quantified from a set of two-dimensional coordinates (landmarks) with tpsDIG 1.40 software (Rohlf 2004). Eight landmarks were established: one type 1 (structures or points defined locally), three type 2 (points located at local curvature maxima), and four type 3 (points distant from the type I landmark; Zelditch et al. 2004; Fig. 2). To remove the nonshape variation (differences because of location, scale, and orientation) from the landmark configurations, a Generalized Procrustes Analysis (GPA) was used (Rohlf and Slice 1990, Rohlf 1999) as implemented in the CoordGen6 program of Integrated Morphometrics Package (IMP) (Sheets 2003) to produce a set of partial Procrustes superimpositions of specimens. The coordinates obtained were transformed into relative warp scores to produce a W matrix (Zelditch et al. 2004), and shape variation between morphotypes was analyzed with a principal component analysis (=relative warp analysis) in PCAGen 6 (Sheets 2003). The change in seminal rod geometric configuration of each specimen was visualized by thin-plate spline deformation grids in PAST 1.95 (Hammer et al. 2001). For evaluation of significant differences between species and morphotypes, the W matrix was analyzed through multivariate analysis of variance (MANOVA) and post hoc pairwise Hotelling's T comparisons. For evaluation of genitalia size differences, an ANOVA test was performed on seminal rod centroid size for each group (i.e., morphotype and species). Centroid size was calculated in PAST 1.95 (Hammer et al. 2001). In addition, the relationship between seminal rod size and shape was analyzed by a Pearson's correlation test between log-centroid size and relative warp one (RW1).

Semilandmark Analysis—Spermatheca. Semilandmarks (arbitrary points along a curvature) were used to capture outline information on spermathecae because of insufficient numbers of well-defined homologous points suitable for landmarks. Semilandmarks may slide along the outline to optimally match corresponding points of other specimens. The application MakeFan6 (Sheets 2003), which places alignment "fans" at equal angular displacements along a curve, was used to ensure consistent placement of the semilandmark points on spermatheca images. A fan of 19 radiating lines was added to all spermatheca images. Radiating lines were of equal angular intervals, and the points where they met the margin of the spermatheca constituted semilandmarks (Bookstein 1997, Zelditch et al. 2004). Both landmarks and semilandmarks were then digitized using tpsDIG version 1.40 software (Rohlf 2004) for, in total, 2 landmarks and 36 semilandmarks for each specimen (Fig. 2). When semilandmarks are digitized at discrete points, a coordinates adjustment is recommended before GPA to minimize the tangential variation of semilandmarks on the spematheca contour. Therefore, the minimum Procrustes distance criterion (Perez et al. 2006) was used for an optimal superimposition with SemiLand 6 (Sheets 2003). To remove the nonshape variation, the landmark–semilandmark configurations of specimens were superimposed with GPA. Shape variation between morphotypes and the change in spermatheca geometric configuration of each specimen were analyzed as it was describe in Landmark Analysis for seminal rod. To evaluate statistical differences in spermatheca shape between morphotypes, the relative warps (W matrix) were analyzed through Hotelling's T test. Genitalia size differences between morphotypes were compared with a Student's *t*-test using spermatheca centroid size of both morphotypes. Centroid size was calculated in PAST 1.95 (Hammer et al. 2001). The relation between spermatheca shape and size was analyzed as it was describe above for seminal rod. Univariate and multivariate tests and plots (seminal rod and spermatheca) were completed using Matlab version 7.8.0.347 for windows (The MathWorks Inc.).

Karyology. The karyology of *D. frontalis* and its sister species has been studied extensively by previous authors (Lanier 1981; Lanier et al. 1988; Salinas-Moreno et al. 1994; Zúñiga et al. 1998, 2002a). Specimens of *D. frontalis* karyotyped by Lanier et al. (1988), Salinas-Moreno et al. (1994), Zúñiga et al. (1998), and Zúñiga et al. (2002a) were a sample of the insects set collected specifically for those studies, which were deposited at Laboratorio de Variación Biológica y Evolución de la Escuela Nacional de Ciencias Bilógicas of Instituto Politécnico Nacional (ENCB-IPN) and University of Nuevo León Campus Linares. To classify the vouchers (20 individuals) and specimens (302 individuals from 13 Mexican locations) of these collections as either MA or MB, we reviewed the presence or absence of prothoraxic striations. In addition, we performed a karyological analysis of MA and MB using live adult specimens from Parque Nacional Lagunas de Montebello, Chiapas (Table 1).

The reproductive system of 20 males and 20 females each of morphotypes A and B were removed under Ringer's solution, and then testes and ovarioles were isolated and fixed in Farmer's solution (3:1 ethanol–acetic acid). Slides were made using the aceto-carmine squash technique, and 100 meiotic figures for all slides were analyzed from these preparations. Chromosomal number was determined by counting bivalent chromosomes in late prophase and metaphase, and meiotic dynamics were examined for possible irregularities at the level of supernumerary chromosomes, irregular associations in bivalents, and changes in the chromosome number. Slides were observed under a phase-contrast microscope at 1,250×, and the best figures were photographed using a Nikon Coolpix 5000 camera.

Molecular Analysis. Total genomic DNA of 10 adult insects from each morphs collected in Parque Nacional Lagunas de Montebello, Chiapas, was extracted and purified (Table 1) using a DNAeasy tissue kit (QIAGEN GmbH, Hilden, Germany). Amplification of an 800-bp fragment of the mitochondrial *cytochrome oxidase I* (COI) gene was carried out using primers TL2-N-3014 (Simon et al. 1994) and Mod-CI-J-2183 (CAACACTTATTTTGATTTTTTGG) designed for

this study. Polymerase chain reaction (PCR) was performed using a Biometra T Gradient thermocycler (Biometra GmbH, Hilden, Germany). Amplification and sequencing of the mitochondrial DNA (mtDNA) COI fragment was carried out as described in Ruiz et al. (2009). To assess whether pseudogenes could have been amplified, a restriction fragment length polymorphism analysis (RFLP) were carried out on fragments amplified (data not shown).

The maximum-likelihood (ML) algorithm implemented in the program aLRT-PHYML (Guindon and Gascuel 2003) was used to infer the phylogenetic relationships among mitochondrial DNA sequences of both morphotypes of *D. frontalis.* Before the ML analysis, we determined an appropriate model of DNA evolution and model parameters using both the Akaike Information Criterion and hierarchical likelihood ratio tests, as implemented in Modeltest v3.7 (Posada and Crandall 1998). Both tests supported the Tamura-Nei model (−LnL 2945.83; Tamura and Nei 1993) with gamma ($G = 0.31$) distributed rate variation and transitions and transvertions proportion ($R = 3.37$). These *G* and *R* values, along with an optimized base frequency, were used in aLRT-PHYML. To estimate nodal support, the approximate likelihood ratio test (aLRT; Anisimova and Gascuel 2006) with the Shimodaira–Hasegawa-like procedure option was used. Two sequences (AF067986 and AY570903) from *D. frontalis* deposited in the National Center for Biotechnology Information (NCBI) were included in our analysis (Kelley and Farrell 1998, Duan et al. 2004). The sequences of *D. approximatus* (AF068000), *D. adjunctus* (AF068001 and AF067992), *D. brevicomis* (AF067999 and AF068002), *D. mexicanus* (AF067988), and *D. vitei* (AFO68004; Kelley and Farrell 1998) were included as outgroups.

Cross-Breeding Experiments. Logs from both infested and uninfested *Pinus oocarpa* Schiede ex Schitdl and *Pinus maximinoi* H. E. Moore were obtained in the Parque Nacional Lagunas de Montebello, Chiapas, Mexico, in 2009 and 2010 and transported to Colegio de la Forntera Sur (=ECOSUR) Tapachula for experimentation (Table 1). Uninfested logs for crossing tests were 30 cm in length and stored under refrigeration (0–10°C) up to 10 d before use. Logs of infested *P. oocarpa* and *P. maximinoi* were enclosed in cloth bags (50 by 70 cm), and emerged beetles were collected daily and housed in plastic petri dishes lined with moist paper towel and held in refrigeration up to 5 d before use in crossing experiments. Uninfested logs were allowed 4 h and beetles 1–2 h, to acclimate to room temperature after removal from refrigeration.

Sperm Transfer. To assess possible prezygotic reproductive isolation between the two morphotypes, sperm transfer among individuals was analyzed for artificial crosses in the laboratory. Replicates were performed for all four possible crosses in both host trees. Attacks were induced on uninfested logs by confining females under a piece of plastic screening within a ≈1-cm-diameter by ≈3-mm-depth pit cut into the outer bark with a cork borer. Within the pit, a ≈1-mm-diameter hole was drilled into the phloem within each pit. Attacks were spaced >6 cm apart on the bark, with three to six attacks per log. Females were confined for 18–24 h before a male was introduced into the enclosure. Pairs were then excised alive from the gallery after 48–72 h. Spermathecae were dissected immediately from females, mounted in water on slides, and examined with phase contrast microscopy at 40× for presence of sperm. To assess whether frequency of insemination differed among the cross types, we performed Fisher exact tests (Zar 2010) on the 2 by 4 contingency table of the number of individuals with or without sperm versus type of cross. All-pairwise Fisher exact tests with a Bonferroni correction were used to contrast the sperm transfer of the different cross types. In addition, we examined the spermathecae of 20 unpaired females (i.e., not introduced into logs) of each morphotype to determine whether some proportion of females used in the crosses might have already been inseminated before pairing.

Gallery and Brood Production. To assess postzygotic reproductive isolation and reproductive success in pairings between individuals of different morphotypes, we repeated the procedures above mentioned of the sperm transfer tests but incubated the logs at room temperature for 20–50 d before dissecting the gallery systems. For each type of cross, egg niches and larval galleries were counted per parent gallery system as approximations of the numbers of eggs laid and hatched, respectively. Egg hatching percentage was estimated as the ratio of larval galleries to egg niches in each gallery system. In addition, the length of each parent gallery was measured.

Variables parental gallery length and larvae per brood (i.e., larval galleries per parental gallery that had a least one larval gallery) were subjected to a square-root transformation to meet the assumptions of parametric statistics, and these data along with egg niches per parent gallery were each evaluated separately by a two-way ANOVA (Zar 2010) with factors cross type (MA♀ × MA♂, MB♀ × MB♂, MB♀ × MA♂, and MA♀ × MB♂) and host species (*P. oocarpa* and *P. maximinoi*). All-pairwise comparisons were carried out with Tukey's test. The variable larvae per niche could not be transformed adequately because of the large numbers of zeros, and therefore host trees were pooled and data were analyzed with a nonparametric one-way Kruskal-Wallis test (Zar 2010) with factor cross type: all-pairwise comparisons among cross types were carried out with Mann-Whitney tests and a Bonferroni correction. Proportions of parental galleries possessing at least one egg niche or larval gallery were separately compared among crosses by means of all Fisher exact tests with Bonferroni correction.

Results

Univariate Character Analyses of Morphotype

Outline of the Frons (OF). (♀♂). Thirteen percent of MB specimens presented a truncated convex frons and the remainder possessed fully convex frons. In MA 15% had truncated convex frons

Table 2. Statistical analyses[a] of continuous characters examined in two morphotypes[b] of *D. frontalis*

Character[c]	MA (μm)	MB (μm)	t	(p)
Length of frontal tubercles	32.78 ± 0.9	33.6 ± 1.4	1596	0.7472
Width of epistomal process	389.5 ± 5.6	510.8 ± 11.0	1502	<0.001
Distance between eyes	678.3 ± 6.3	831.5 ± 18.3	1277	<0.001
Width of left eye	201.1 ± 1.2	241.6 ± 3.5	1117	<0.001
Length of head–pronotum	1383.8 ± 20.8	1663.4 ± 26.2	1970	<0.001
Pronotum length	812.6 ± 11.0	978.1 ± 14.8	1867	<0.001
Pronotum width	1186.1 ± 10.0	1396.4 ± 31.6	1655	<0.001
Elytra length	2004.5 ± 18.5	2261 ± 34.7	2275	<0.001

[a] *t*-statistic and *P* value of Student's *t*-test.
[b] MA, morphotype A; MB, morphotype B.
[c] Illustrated in Fig. 1k.

whereas the 85% had a fully convex frons. Morphotypes did not differ significantly (U = 63.9; Z = −0.14; $P = 0.491$).

Number of Rows of Squamiform Plates on the Eighth Tergite (RSP). (♀). In both morphotypes, the mean values for this character were similar (MB = 18 ± 0.4 SE; MA = 16 ± 0.6 SE), but differed statistically (U = 190.5; Z = −2.705; $P \leq 0.05$).

Acute Projections on the Posterior Edge of Squamiform Plates at the Median Proximal Area of The Eighth Tergite (AP). (♀). All MA females possessed plates with uniformly short ornamentations and all MB females had plates with irregularly sized ornamentations. The morphotypes differed significantly for this character (U = 0; Z = −8.055; $P \leq 0.001$).

Shape of the Cornu of the Spermatheca (SCS). (♀). Ninety percent of MB females possessed an ovate cornu whereas 10% had a round cornu. MA displayed the reverse trend, with 34% of the females possessing an ovate cornu and the 66% a round cornu. The morphotypes differed significantly (U = 229; Z = −2.358; $P = 0.019$).

Number of Striae on Spermatheca (NSS). (♀). In both morphotypes, the mean values for this character (MB = 16.7 ± 0.7 SE; MA = 15.6 ± 0.7 SE) did not differ statistically between morphotypes (U = 348.5; Z = −1.087; $P = 0.2769$).

Density of Striae at the Proximal Region of Spermatheca's Nodulus (DS). (♀). One hundred percent of the all MB females had no-agglomerate striations. In the MA females the 55% had aggregate striations and the 45% no-agglomerate striations. Mann-Whitney test showed statistically significant differences between morphotypes (U = 169; Z = −3.898; $P \leq 0.001$).

Proportion of the Spermatheca Covered by Striae (PSS). (♀). Sixty-five percent of the MA had spermatheca with a fourth part its surface covered with striations, while the 35% had more than half of the surface covered with striations. In the MB the 100% of the specimens had more than half of the surface covered by striations. Morphotypes possessed statistically significant differences by Mann-Whitney test (U = 184; Z = −4.084; $P \leq 0.001$).

Shape of Seminal Rod Lobe (SSR). (♂). All MA specimens showed a convex shape of lobe and 100% of the MB had a plane or slightly convex lobe. The morphotypes differ significantly (U = 0; Z = −14.59; $P \leq 0.001$).

Continuous Characters. (♀♂). For all continuous characters, the MB possessed higher mean values than MA (Table 2). Student's *t*-test indicate statistically significant differences between morphotypes for all body measures analyzed (i.e., EPW, DE, WE, LHP, PW, PL, and E) except the LFT.

Multivariate Analyses

Comparison of MA and MB Males. PCoA and cluster analysis were conduced with 99 MA and 65 MB using characters (EL, LHP, PL, PW, DE, WE, EPW, OF, SSR, and PAE). The first three principal coordinates together explained 73.57% of total variation (PCo1, 65.2%; PCo2, 4.99%; and PCo3, 3.38%). A scatterplot of the two first components (PCo1 and PCo2) showed discrete and distinct phenotype separation corresponding to each morphotype (Fig. 3a). In the cluster analysis dendrogram, two groups corresponding to each morphotype were obtained, with bootstrap values of 74% for the MA group and 73% for the MB group. In the MB cluster, two subgroups were present with bootstrap values <50%, one with specimens exclusively from Oaxaca state localities and the other with specimens from both Chiapas and Oaxaca states (Fig. 3a). The tree had a high cophenetic correlation index ($r = 0.917$; $P \leq 0.05$).

Comparison of MA and MB Females. PCoA and cluster analyses were carried out with 45 MA and 25 MB females of morphotype using characters (EL, LHP, PL, PW, DE, WE, EPW, OF, RSP, SCS, AP, NSS, DS, PSS, and PAE). The first three principal components together explained 62.66% of total variation (PCo1, 43.87%; PCo2, 12.28%; and PCo3, 6.51%). Scatter plots of the first two coordinates (PCo1 and PCo2) showed discrete and distinct clusters corresponding to each morphotype (Fig. 3b).

In the female dendrogram, two groups were obtained corresponding to the two morphotypes, with a bootstrap value of 71% for the MA group and 86% for the MB group (Fig. 3b). Within both clusters, no geographical subgroups were found, and the cophenetic correlation index was 0.844 ($P \leq 0.05$).

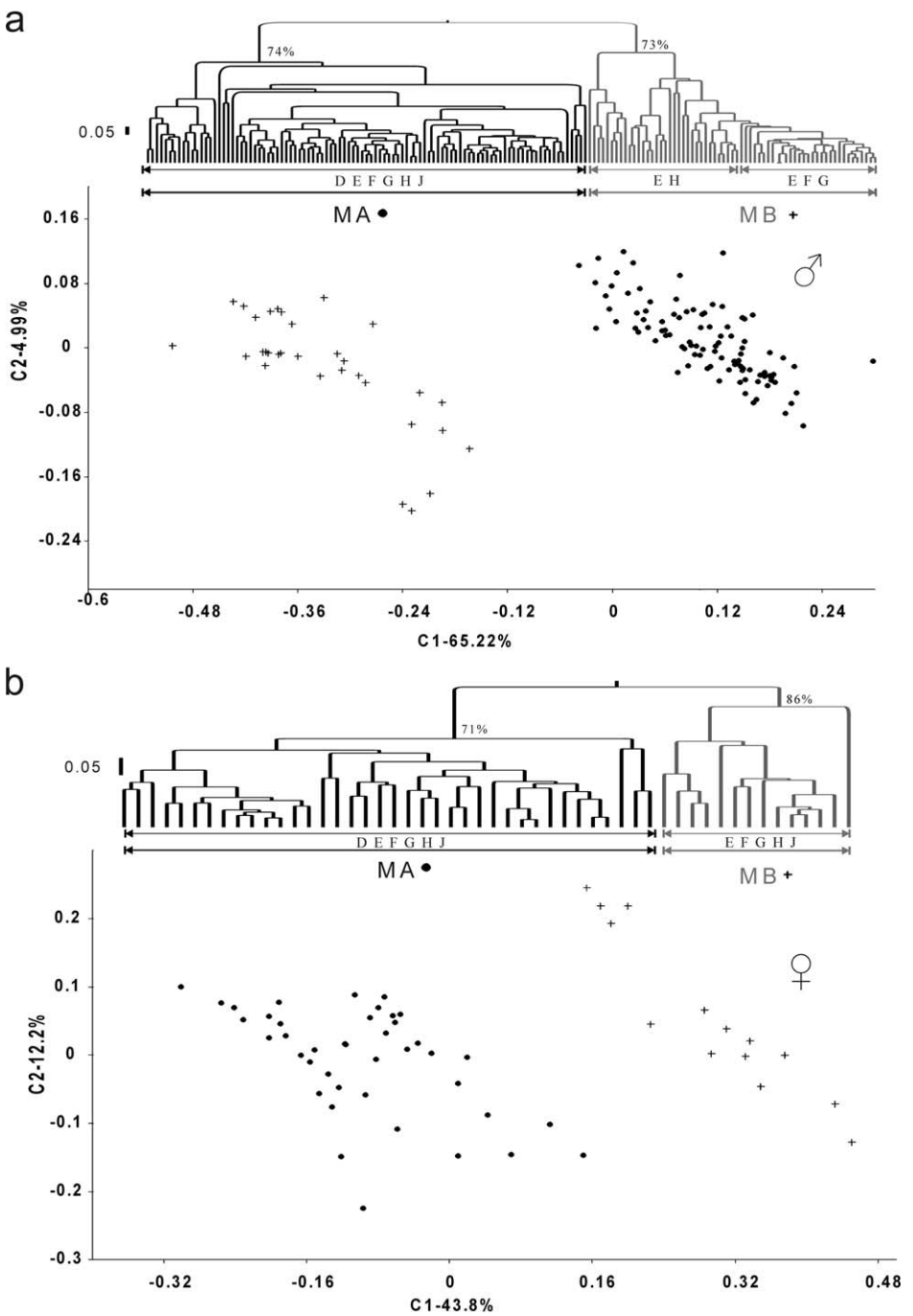

Fig. 3. Scatter plots from principal coordinate analysis and dendrograms from cluster analyses of morphological characters of both morphs of *D. frontalis*: (a) males and (b) females. Bootstrap values (1,000 pseudoreplicates) are indicated at the nodes and capital letters under the dendrograms indicate the populations analyzed from Table 1. MA, morphotype A; MB, morphotype B.

Comparison of Both Morphotypes with *D. mexicanus* and *D. vitei*. PCoA and cluster analyses were carried out with 164 MA, 66 MB, 170 *D. mexicanus*, and 10 *D. vitei* using characters (EL, LHP, PL, PW, DE, WE, SSR, and PAE). Owing to shape of posterior margin of the seminal rod's lobe from *D. mexicanus* and *D. vitei* being concave, the charcater (SSR) for these species was coded as a different character state (3) for multivariate analyses. The first three principal coordinates together explained 72.67% of total variation

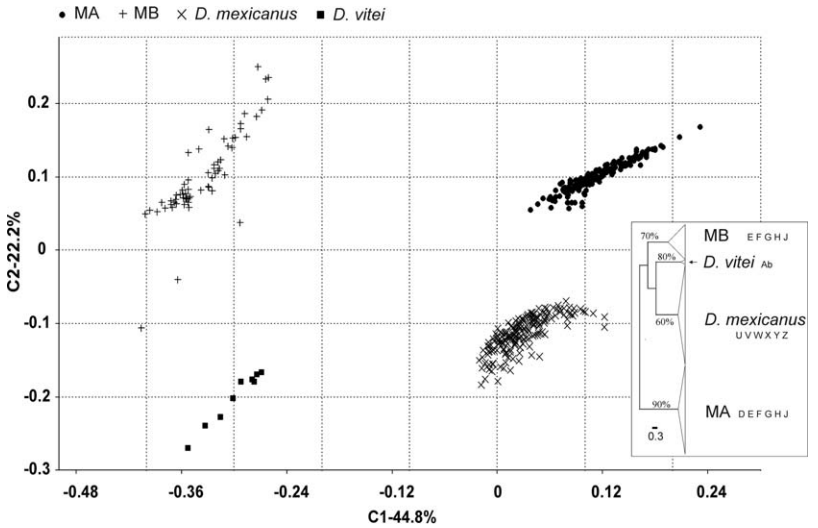

Fig. 4. Scatter plot from principal coordinate analysis and dendrogram from cluster analyses of morphological characters of both morphotypes of *D. frontalis*, *D. mexicanus*, and *D. vitei*. Bootstrap values (1,000 pseudoreplicates) are indicated at nodes and capital letters adjacent to the dendrogram indicate the populations analyzed (Table 1). MA, morphotype A; MB, morphotype B.

(PCo1, 44.83%; PCo2, 22.6%; and PCo3, 5.24%). The scatterplot of the first two coordinates showed discrete and distinct phenotype clusters corresponding to the four groups analyzed (Fig. 4). The dendrogram had a cophenetic correlation index of 0.994 ($P \leq 0.05$) and the topology showed four groups supported by a bootstrap values >60% that correspond with the two morphotypes and two species. Both morphotypes were clearly separated, and MB was more closely related to the cluster formed by *D. mexicanus* and *D. vitei* than to MA (Fig. 4).

Geometric Morphometrics

Landmark Analysis—Seminal Rod. The GPA-superimposed configuration of 131 MA, 114 MB, 30 *D. mexicanus*, and 12 *D. vitei* seminal rods indicated that the positional range of variation of each landmark was different. The landmarks that displayed highest variation were those describing the seminal rod spine and lobe shape. In the relative warp analysis, the first three relative warps explained >73% of variation (RW1, 46.3%; RW2, 19.3%; and RW3, 8.5%). The two-dimensional scatterplot of the first two relative warps showed four discrete and distinct clusters corresponding to each species and morphotype (Fig. 5a). The deformations in RW1 are associated to the invagination and evagination of the posterior margin of the lobe and the relative size of the spine (Fig. 5a) and in RW2 the changes are related to the degree of inclination of the spine and elongation of the lobe (Fig. 5a).

A MANOVA on relative warp components of shape revealed statistically significant differences ($\lambda_{Wilks} = 0.0034$; $F_{33, 680} = 263.2$; $P \leq 0.001$) among morphs and species groups. Pair-wise Hotelling's T tests supported statistically significant differences in shape for six comparisons: MA vs. MB ($T = 2503$; $F = 138.4$; $P \leq 0.001$), MA vs. *D. mexicanus* ($T = 3.871 \times 10^7$; $F = 1.512 \times 10^5$; $P \leq 0.001$), MA vs. *D. vitei* ($T = 3.483 \times 10^{17}$; $F = 4.582 \times 10^{15}$; $P \leq 0.001$), MB vs. *D. mexicanus* ($T = 1.71 \times 10^5$; $F = 668.9$; $P \leq 0.05$), MB vs. *D. vitei* ($T = 5.19 \times 10^{15}$; $F = 8.11 \times 10^{13}$; $P \leq 0.001$), and *D. mexicanus* vs. *D. vitei* ($T = 7.098 \times 10^4$; $F = 1109$; $P \leq 0.001$).

On average, MA and *D. mexicanus* possessed the highest centroid size values (652.2 ± 17.6 SE and 622.7 ± 30 SE, respectively) while *D. vitei* and MB had smaller sizes (525 ± 21.7 SE and 584.4 ± 39.5 SE, respectively). Significant centroid size differences were detected among morphs and species (ANOVA, $F_{3, 244} = 8.037$; $P \leq 0.05$). However, Tukey tests detected significant differences only between *D. vitei* and the remaining groups ($Q = 3.83$; $P \leq 0.007$). Pearson correlation analysis between centroid size and the first relative warp was not significant ($r = 0.198$; $P \geq 0.05$), suggesting that size was not an important factor influencing shape differentiation.

Semilandmark Analysis—Spermatheca. The superimposition configuration of 35 MA and 25 MB spermathecae showed that the semilandmarks with highest variation are those describing the curvature of the spermatheca, nodulus width, and cornu shape. The first three components of the relative warp analysis explained 66.6% of shape variation (RW1, 39.5%; RW2, 17.4%; and RW3, 9.7%). A two-dimensional scatterplot of these relative warps revealed two distinct but overlapping clusters corresponding to each morphotype (Fig. 5b). The deformations in RW1 were correlated with the degree of concavity of the spermatheca and nodulus width (Fig. 5b) and in RW2, with the length of the nodulus and cornu (Fig. 5b).

Hotelling's T test on relative warp components indicated statistically significant differences between

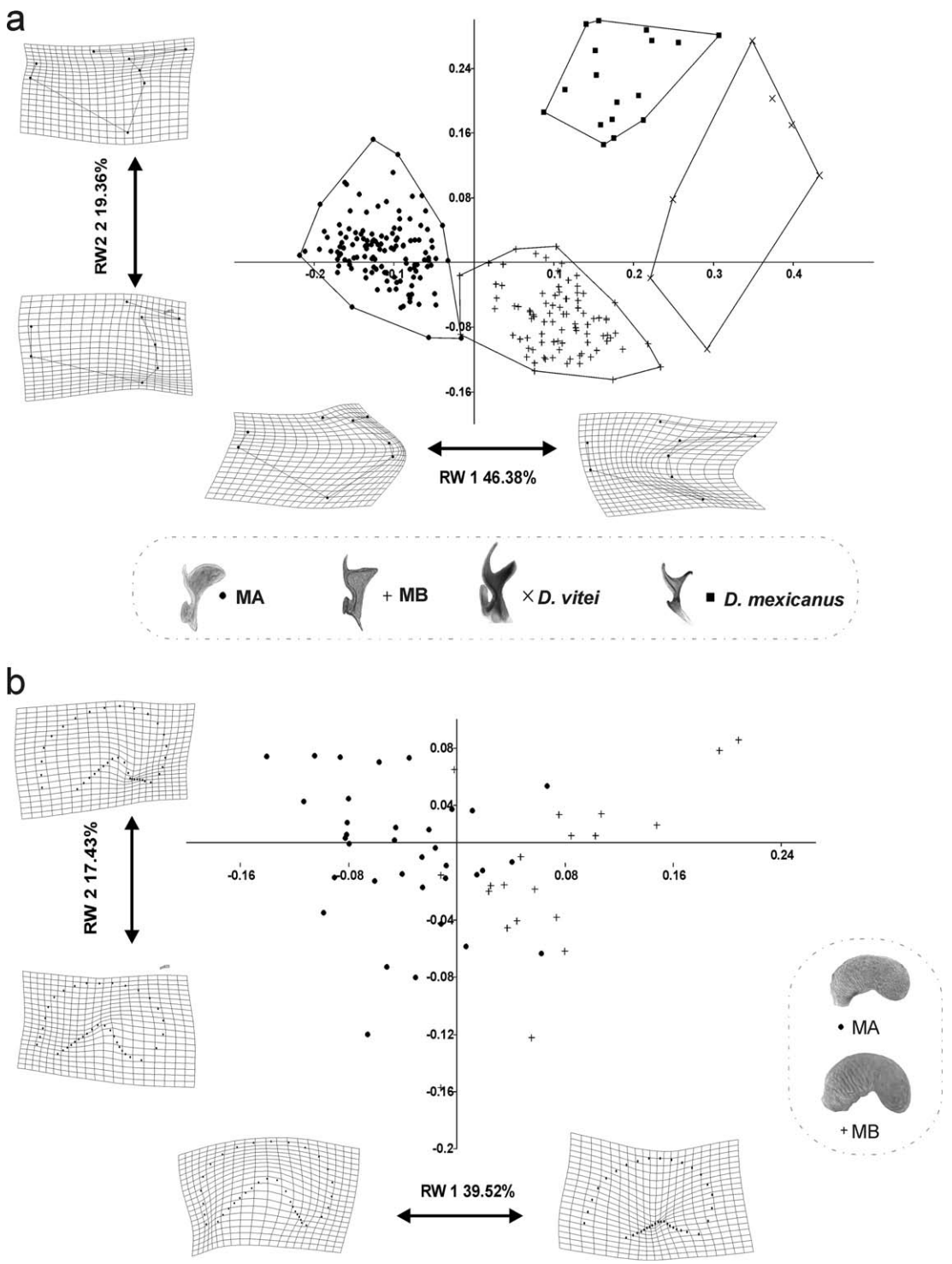

Fig. 5. Scatter plots of the first and second relative warps: (a) male seminal rod analysis of both morphotypes of *D. frontalis*, *D. mexicanus*, and *D. vitei* and (b) female spermathecae analysis of both morphotypes of *D. frontalis*. Changes in genitalic structures along both axes are shown by landmark configurations in thin-plane-spline deformation grids. MA, morphotype A; MB, morphotype B.

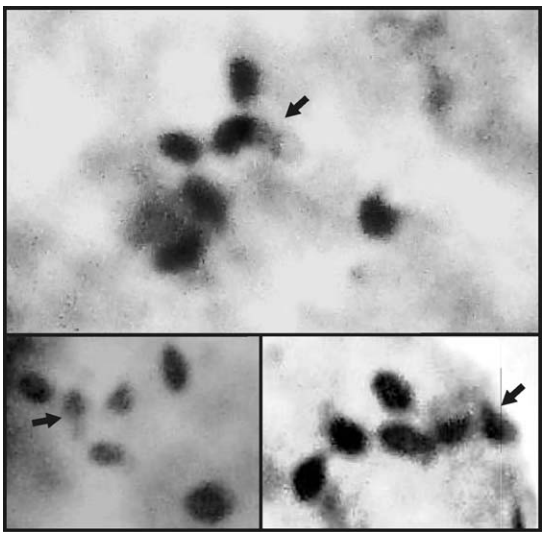

Fig. 6. Spermatocytes of *D. frontalis* morphotype B in prometaphase I with six chromosome pairs 1,000×. Arrow indicates the associated sexual pair, Xyp.

the spermatheca shape of the two morphotypes ($T = 2.81 \times 10^{17}$; $F = 2.89 \times 10^{14}$; $P \leq 0.001$). MB had greater average spermatheca centroid size than MA (1705.3 ± 26 and1579.9 ± 55.4, respectively; $t = 2.93$; $P \leq 0.05$). The Pearson correlation between centroid size and the first relative warp was significant ($r = 0.5$; $P \leq 0.05$), suggesting that size is an important factor influencing shape differentiation.

Karyology. Karyotyped vouchers and specimens collected for kariological studies did not show the presence of prothoraxic striations; therefore, we assumed that the karyotype previously reported for *D. frontalis* corresponded to morphotype A. The chromosomal number of MB specimens was obtained by analyzing figures corresponding to meiosis I (prophase, metaphase, and anaphase) in testes and ovarioles. The diploid chromosome number (2n) of MB was 12 chromosomes, with a meiotic formula of five autosome pairs plus a sexual pair with an Xyp configuration (5AA + Xyp; Fig. 6). Examination of figures did not reveal atypical chromosome dynamics, presence of supernumerary chromosomes, or chromosome alterations such as translocations or inversions. The meiotic formula for MA was 7AA + Xyp; the same formula was reported for *D. frontalis* in previous studies (data not shown).

Molecular Analysis. Ten partial sequences of each morphotypes of ≅845 bp of CO1 were obtained. We did not observed double peaks, nonsynonymous mutations, indels, frameshifts, or additional stop codons, which suggest a low probability of having sequenced nuclear copies (nuclear mitochondrial DNA [NUMTs]). After the manual edition of the sequences, a fragment of 786 bp was used in our analysis (GenBank KC855256–62). This fragment is located between positions 2242 and 3028 in the *Drosophila yakuba* mitochondrial genome. The recovered phylogenetic tree showed a clear separation among species and between both morphotypes (Fig. 7). Cluster I corresponded to *D. adjunctus*, *D. brevicomis*, and *D. approximatus*, with the latter two segregating as sibling species. Cluster II contained sister taxa *D. frontalis*, *D. mexicanus*, and *D. vitei*. All *D. frontalis* (both morphotypes) formed a single group with high nodal support (100%), and morphotype B was recovered as a single group with 99% support. The two sequences from *D. frontalis* deposited in the NCBI (specimens from Texas and Arizona) were integrated within of the MA group.

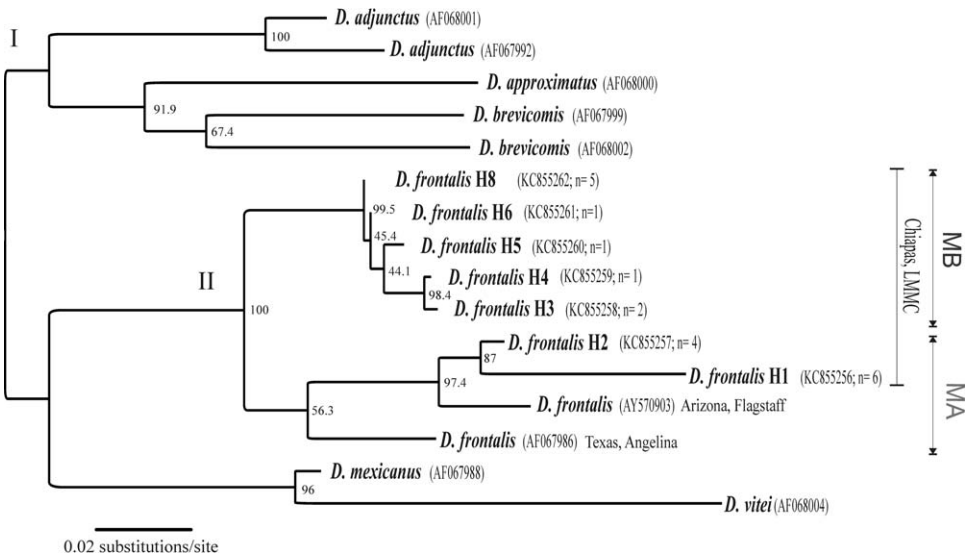

Fig. 7. Phylogenetic tree resulting from ML analysis of cytochrome oxidase I (COI). The model with the best fit for nucleotide substitution was TrN-G-R. Support values at nodes were derived from 1000 pseudoreplicates. *n* = number of individuals sequenced with similar haplotype (H). MA, morphotype A; MB, morphotype B.

Table 3. Percentage (inseminated/total) of inseminated females resulting from crosses of two morphotypes[a] of *D. frontalis* on logs of two different host species[b]

Cross type	*P. oocarpa*	*P. maximinoi*	Total
MA♀ × MA♂	88% (21/24)a	n/a	88% (21/24)a
MB♀ × MB♂	85% (11/13)ab	88% (7/8)a	86% (18/21)a
MB♀ × MA♂	50% (7/14)ab	57% (8/14)a	54% (15/28)ab
MA♀ × MB♂	36% (4/11)b	25% (1/4)a	33% (5/15)b

[a] MA, morphotype A; MB, morphotype B.
[b] Cross types associated with different letters differed significantly in the proportion of inseminated females (all-pairwise Fisher exact tests with Bonferroni correction for the number of contrasts).

Cross-Breeding Experiments

Sperm Transfer. Spermathecae of 20 unpaired MA and MB females from the same pool collected for use in the crossing experiments were dissected and none contained sperm. In total, 24 MA♀ × MA♂, 21 MB♀ × MB♂, 28 MB♀ × MA♂, and 15 MA♀ × MB♂ crosses were performed in which the male entered the female gallery within 24 h and the female was recovered alive afterward (Table 3). MA♀ × MA♂ crosses were obtained only on *P. oocarpa*, whereas the other three cross categories were performed on both *P. oocarpa* and *P. maximinoi*. Fisher exact test showed a significant association between the insemination rate and the cross type with both hosts pooled ($P < 0.001$) as well as for *P. oocarpa* alone ($P = 0.004$). Crosses within morphotype generally had a higher frequency of sperm transfer than crosses between morphotypes. With both trees included in the data, MA♀ × MB♂ crosses had a significantly lower insemination rate (33%) than either homotypic cross (88 and 86%), whereas MB♀ × MA♂ crosses (54%) did not differ significantly in insemination rate from any other cross type (Table 3). For the three cross types attempted on both pine hosts, insemination frequencies were similar for both hosts.

Gallery and Brood Productions. In total, 44 MA♀ × MA♂, 47 MB♀ × MB♂, 32 MB♀ × MA♂, and 30 MA♀ × MB♂ pairings were performed (Table 4), and in all cases males entered the female gallery entrance within 24 h. Significant differences were detected among cross types in the average length of the parent galleries ($F = 4.17$; df = 3, 145; $P = 0.007$), the numbers of egg niches per parent gallery ($F = 3.80$; df = 3, 145; $P = 0.012$), the number of larvae per brood ($F = 6.40$; df = 3, 90; $P < 0.001$), and the egg hatch rate (i.e., larval galleries per egg niche; $H = 50.5$; df = 3; $P < 0.001$). Likewise, there were significant differences among cross types in the proportion of pairings that produced larvae (Fisher exact test, $P < 0.001$), but not egg niches (Fisher exact test, $P = 0.19$). Both heterotypic crosses produced a significantly lower proportion of larvae-producing parent galleries and had a significantly lower egg hatch rate than either homotypic cross. The MA♀ × MB♂ crosses produced significantly shorter parental galleries and significantly fewer larvae per brood than either homotypic cross and fewer egg niches per parent gallery than the MA homotypic crosses. However, the reciprocal MB♀ × MA♂ crosses did not differ significantly from any of the other cross categories in these three measurements. There was no evidence that host species influenced the observed differences in reproductive success among the crosses, as there was not a significant interaction detected between cross type and host species for the variables length of parent gallery, egg niches per parent gallery, or larvae per brood.

Discussion

The present integrative approach augments and corroborates published biochemical evidence (Sullivan et al. 2012) indicating the existence of two distinct morphs within *D. frontalis*. The differentiation of morphotypes A and B is supported by several independent data sets: external morphological characters, shape of seminal rod and spermathecae, karyological analysis of the MB, phylogenetic analysis of mtDNA COI sequences, and crossing experiments between morphotypes. Our analyses strongly suggest that the MB is a distinct taxon that is distinguished from MA (*D. frontalis* sensu stricto) both genetically and morphologically and from other species within the *D. frontalis* complex.

Characters Analyzed. The evaluation of eight discrete characters showed that five (PSS, DS, AP, SCS, and SSR) were also useful for separating the *D. frontalis* morphotypes similarity to PAE. From such attributes, three could be used as diagnostic characters for *D. frontalis* morophotype B, because they distinguish it from *D. frontalis* morphotype A, *D. mexicanus*, and *D. vitei* by the presence of striae on preepisternal area (PAE), the irregular size of ornamentations of the plates on eighth tergite of females (AP), and the shape of seminal rod (SSR). Although with some experience the morphs can be reliably distinguished by the shape of the seminal rod (plane or convex), the shape variation of this character in MB should be used with caution because the lobule is sometimes slightly convex. Spermathecal characters PSS, DS, and SCS showed overlap of their states between morphotypes; however, these features can be useful in combination with nonoverlapping characters (i.e., AP and SSR) for identification of *D. frontalis* morphotypes, as their average states differed significantly between them.

The statistical analysis of continuous attributes (DE, EL, EPW, LFT, LHP, PL, PW, and EW) also allowed separation of morphotypes. In general terms, MB presented higher mean values than MA for these measurements. In other cases of sibling species in the genus and in other scolytines, quantitative measures have sometimes been used for species separation. Examples include pronotal width in *Dendroctonus ponderosae* Hopkins and *Dendroctonus jeffreyi* Hopkins (Lanier and Wood 1968) and in species within the *frontalis* complex (Lanier et al. 1988), relative length of abdominal sternites in *Dendroctonus valens* LeConte and *Dendroctonus terebrans* Olivier (Pajares and Lanier 1990), and five different continuous char-

Table 4. Results of pairing experiments with *D. frontalis* morphotypes[a]

Cross type	Total pairings	Length of parent gallery (cm)[b,c,g]	Parent galleries with egg niches[d,g]	Egg niches per parent gallery[b,g]	Parent galleries with larvae[e,g]	Larvae per brood[b,c,f,g]	Larvae per egg niche[f,g]
MA♀ × MA♂	44	43.1 ± 3.5a	42a	66.8 ± 5.9a	41a	39.0 ± 3.8a	0.519 ± 0.032a
MB♀ × MB♂	47	38.9 ± 3.4a	44a	54.1 ± 6.5ab	38a	38.3 ± 4.3a	0.533 ± 0.040a
MB♀ × MA♂	32	28.7 ± 3.0ab	27a	38.3 ± 6.7ab	8b	29.8 ± 11.5ab	0.114 ± 0.044b
MA♀ × MB♂	30	26.5 ± 3.9b	25a	36.1 ± 5.9b	11b	12.6 ± 4.5b	0.134 ± 0.050b
ANOVA							
$P_{(\text{crosses})}$		0.007		0.012		<0.001	<0.001
$P_{(\text{host})}$[h]		0.49		0.52		0.76	n/a
$P_{(\text{interaction})}$		0.11		0.052		0.19	n/a

[a] MA, morphotype A; MB, morphotype B.
[b] Means associated with the same letter did not differ significantly (Tukey's test; $\alpha = 0.05$).
[c] Both the two-way ANOVA and all-pairwise tests were performed on square-root transformed data.
[d] Numbers of parent galleries with at least one egg niche present. Cross types associated with a different letter differed in the proportion of parent galleries with or without niches (all-pairwise Fisher exact tests with Bonferroni correction; corrected $\alpha = 0.0083$).
[e] Numbers of parent galleries with at least one larval gallery evident. Cross types associated with a different letter differed in the proportion of parent galleries with or without larval galleries (all-pairwise Fisher exact tests with Bonferroni correction; corrected $\alpha = 0.0083$).
[f] Mean numbers of larval galleries per parent gallery that produced live brood (i.e., that had at least one larval gallery).
[g] Means associated with the same letter did not differ significantly (all-pairwise Mann-Whitney tests with Bonferrroni correction; corrected $\alpha = 0.0083$). Results of one-way ANOVA on ranks (Kruskal-Wallis test).
[h] Crosses were performed in similar numbers on *P. oocarpa* and *P. maximinoi.*

acters in *Tomicus destruens* and *Tomicus piniperda* (L.) (Faccoli 2006).

Likewise, multivariate analysis of quantitative and qualitative data agreed with the recovery of two discrete groups corresponding to the morphotypes. Robustness of these clusters was demonstrated when *D. vitei* and *D. mexicanus* (species with a high morphological similarity with *D. frontalis*) were incorporated into the analysis and the inclusion of these additional samples did not disrupt the original clusters corresponding to each morphotype. Multivariate analysis has been used extensively to identify and delimit morphological and genetic variation within and between species (Doyen 1973, Doyen and Slobodchikoff 1974, Mutanen 2005, Adeleke et al. 2008, Jeffrey et al. 2008, Padial and De la Riva 2009, Thorpe 2010).

Shape Variation of Genitalia. Geometric morphometric analysis of genitalic structures (seminal rod and spermatheca) supports separation of the morphotypes and also the separation of them with *D. mexicanus* and *D. vitei.* Variation in seminal rod shape was concentrated in two specific sites: the lobe and the spine (Fig. 5a), and was congruent with discrete seminal rod character (i.e., SSR; posterior outline of lobe either convex and plane or slightly convex) that allowed discrimination of the morphotypes.

Seminal rod shape is a key character used for identification of *Dendroctonus* species (Vité et al. 1975, Wood 1982, Lanier et al. 1988); however, a rigorous assessment of the taxonomic potential of this structure had never been carried out. The current study shows that analysis morphometric landmarks increase the discriminatory value of seminal rod morphology for taxonomic purposes, particularly in situations where seminal rods cannot easily be reliably discriminated, as is the case with the *D. frontalis* morphotypes.

As with the seminal rod, sites of greatest shape variation within the spermatheca indicated by geometric morphometrics were congruent with the character states identified to categorize variation within this structure (nodulus width, curvature of spermatheca, and shape of cornu; Fig. 5b). The discriminatory power of the spermatheca shape was lower than that for the seminal rod, because morphometric data from the spermatheca produced merely partial separation of the morphotypes. Although the shape and number of striae on the surface of the spermatheca have been used as useful characters for separating *D. frontalis* and *D. mexicanus* (Rios-Reyes et al. 2008), results of the current study indicate that spermatheca shape within the *D. frontalis* morphotypes is highly variable and consequently of limited value for distinguishing them.

Chromosome Analysis. The chromosome number of the MB was different from that previously reported for specimens of *D. frontalis* (MA; Lanier et al. 1988; Salinas-Moreno et al. 1994; Zúñiga et al. 1998, 2002a), subsequently determinated to be MA. In addition, we confirmed that the chromosome number previously reported for *D. frontalis* was identical to that of MA specimens collected in the same location as the karyotyped MB. This represents additional evidence that the two morphotypes are taxonomic entities. The chromosome number of MB is identical to other species in the *frontalis* complex including *D. approximatus*, *D. brevicomis*, and *D. mexicanus* (Lanier 1981). The sexual pair of MB has a Xy_p configuration that is unlike the neo-XY configuration of *D. approximatus* and *D. brevicomis* (Lanier et al. 1988, Zúñiga et al. 2002a) but is possessed by *D. mexicanus.* However, differences in external and seminal rod morphology (Figs. 4 and 5) as well as COI sequences (Fig. 7) between MB and *D. mexicanus* discount the possibility that MB and *D. mexicanus* are the same taxon.

It is possible that the karyotype observed in the MB specimens could represent polymorphism within *D. frontalis;* however, it is unlikely that viable and fertile offspring would be produced because of the unbalanced combinations of chromosomes resulting from the mixture of karyotypes $7II + Xy_p$ and $5II + Xy_p$. The

resulting infertility would generate strong selection against such pairings. Furthermore, chromosomal polymorphisms have not previously been detected in any species of *Dendroctonus* (Lanier 1981, Lanier et al. 1988), despite extensive chromosomal sampling of certain species in their geographic range (Salinas-Moreno et al. 1994; Zúñiga et al. 1998, 2002a).

Molecular Analysis. The COI-based tree of species in the *frontalis* complex is consistent with the COI-based phylogeny produced by Kelley and Farrell (1998), with the addition that the two *D. frontalis* morphotypes formed distinct groups within the group of *D. frontalis.* The formation of two groups, one integrated by the MB sequences and the other by the MA sequences derived from sympatric populations (Chiapas), supports the presence of two different terminal taxa. The tree also shows a clear separation of these taxa with respect to *D. vitei* and *D. mexicanus.*

Numerous studies have debated the utility of mtDNA sequencing alone for identifying species, corroborating described species, and recognizing cryptic species in groups with high morphological similarity. In this sense, the COI mtDNA differentiation level found between MA and MB is concordant with morphological differences observed; results similar were documented by Ruiz et al. (2009) for two subspecies from *Dendroctonus pseudotsugae* Hopkins. However, as COI sequences from the small number of specimens examined in this study are likely not representative of the potential variability of both morphotypes, and considering the wide distribution range of *D. frontalis* (MA; Clarke and Nowak 2009) and *Dendroctonus* sp. nov. (MB; F.A.T., unpublished data), caution must be taken regarding the use of mtDNA in the identification of both morphotypes until a complete assessment of COI molecular variation is completed.

Crosses. Our tests to evaluate interbreeding potential between the morphotypes support the hypothesis that they are distinct biological entities. Significantly higher rates of insemination in homotypic crosses compared with at least one of the two heterotypic crosses implies the existence of at least partial prezygotic reproductive isolation barriers between the morphotypes. Incompatible morphology of the genitalia (as suggested by the significant differences in seminal rod morphology between morphotypes) would have been one potential barrier to insemination, but behavioral incompatibility (e.g., unsuccessful courtship) and other physical incompatibilities could also have led to a failure of pairings to copulate or transfer sperm. Similarly, evidence of significantly lower egg hatch for both heterotypic crosses suggests the existence of postzygotic barriers to reproduction, perhaps caused by the differing chromosome formulas of the two morphotypes. Reduced reproductive success by the heterotypic crosses was likewise implied by shorter average parental galleries, a lower frequency of brood production, and smaller broods. Although it was apparent that heterotypic crosses were capable of producing viable F1, we could not ascertain whether these were fertile, as brood of all cross types died before reaching adulthood. Nonetheless, differences in parental chromosome number between morphotypes imply that surviving F1 brood of heterotypic crosses would likely have been sterile.

Forced heterospecific pairings between described members of the frontalis complex have been reported to produce parent galleries with egg niches; however, there are no confirmed reports of such crosses producing F1 brood (Vité et al. 1974, Lanier et al. 1988). Nevertheless, selective pressures because of lower reproductive success of heterotypic crossings by the *D. frontalis* morphotypes should support acquisition and maintenance of traits that reduce incidence of heterotypic pairs. Although it was possible in the laboratory to force or induce beetles of different morphotypes to pair transfer sperm and produce larval brood (albeit at lower rates than homotypic crosses), extensive dissections of parental galleries on pines simultaneously infested by both species did not showed incidences of heterotypic pairs (A.N. and B.T.S., unpublished data). Furthermore, differences in the composition of aggregation and mate location pheromones (Sullivan et al. 2012), spatial partitioning of the host (A.N. and B.T.S., unpublished data), and possible differing times of arrival on the host may provide important mechanisms of prezygotic reproductive isolation in nature that would have been absent in our laboratory studies.

Therefore, we conclude that the two morphotypes of *D. frontalis* are species distinct from each other as well as from other described species in the *D. frontalis* species complex. The zone of sympatry of these two species appears to include at least portions of Nicaragua, Guatemala, Honduras, Belize, and the southern Mexican states of Michoacan, Oaxaca, and Chiapas (F.AT. and G.Z., unpublished data). Morphotype B insects have not been identified by the authors in collections from elsewhere in the range of *D. frontalis,* which includes Sierra Gorda and Sierra Madre Oriental (Salinas-Moreno et al. 2004), Arizona, and the southeastern United States. Specimens examined outside the sympatric zone appear to be entirely MA, which is congruent with both 1) the identical chromosome number from MA in the sympatric zone and *D. frontalis* in U.S. populations and, 2) The COI-tree topology that grouped MA from Chiapas with *D. frontalis* collected in United States. Because allopatric zone of morphotype A includes the type locality of *D. frontalis* ('Carolina'; Wood 1982), we conclude that MA is classified appropriately as *D. frontalis* whereas MB represents an undescribed species. Although these two taxa often coexist on the same individual hosts, studies suggest that they possess different colonization behaviors that might reduce competition. Adult *D. frontalis* (MA) emerge from coinfested trees earlier than *Dendroctonus* sp. nov. (MB), and *D. frontalis* focus their attacks primarily on the upper and midbole, whereas *Dendroctonus* sp. nov. primarily infest the bottom 3 m of the stem (Moreno 2008, unpublished data). Such differences suggest that the taxa occupy different ecological niches, which may promote their coexistence under conditions of syntopy (Rivas 1964).

One important implication of our results is that the bark beetle outbreaks currently attributed to *D. frontalis* in regions where *Dendroctonus* sp. nov. occurs may be caused instead by this latter species or by the combined activity of both *D. frontalis* and *Dendroctonus* sp. nov. Revised procedures for evaluation of bark beetle infestations in the Central American region will be necessary to assure that causative agents of these outbreaks are correctly identified and that appropriate management procedures may be developed and implemented.

Acknowledgments

We thank Juan Cruz, Efraín Santiago, and Francisco Bonilla (Comisión Nacional Forestal México), Juvencio Hernández and Olivia Maldonado (Secretaria de Desarrollo Agropecuario Forestal, Pesca y Acuacultura, Oaxaca, Mexico), Roberto Castellanos (Parque Nacional de Lagunas de Montebello, Mexico), and Benjamin Moreno (Colegio de la Frontera Sur-ECOSUR) for field and lab assistance and for providing insects specimens. Collection specimens were loaned by Luis Gerardo Cuellar Universidad de Nuevo León Campus Linares. The project was funded by U.S. Department of Agriculture Forest Service Southern Research Station cooperative agreements 04-IC-11330129-131 and 07-IC-11330129-056 and partially by the Comisión Nacional Forestal (Project 69539). F.A.T. was member of Programa Institucional de Formación de Investigadores del Instituto Politécnico Nacional (PIFI-IPN); F.A.T. and A.N. were Consejo Nacional de Ciencia y Tecnología fellows (267436 and 256629, respectively).

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Received 5 April 2013; accepted 26 September 2013.