Agricultural and Forest Entomology (2005) **7**, 87–94

Genetic variation and origin of red turpentine beetle (*Dendroctonus valens* LeConte) introduced to the People's Republic of China

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Abstract

1 The red turpentine beetle, *Dendroctonus valens* LeConte, is a recent New World introduction to the People's Republic of China. An outbreak of these beetles has infested over 0.5 million hectares of pine forests.
2 Efforts are underway to suppress this outbreak using biological control measures. However, the wide distribution in the native range of *D. valens* suggests regional variation of the beetle's biology, predators, and parasitoids. Thus, knowledge of the origin of these beetles can help devise precise and effective control measures.
3 A portion of the mitochondrial cytochrome oxidase subunit I gene was sequenced for 218 individuals from 32 populations throughout the native range of *D. valens* and in China.
4 Haplotype diversity was high. A total of 131 haplotypes were found and Jukes–Cantor corrected nucleotide difference ranged from 0 to 16%. Haplotype diversity ranged from 0.53 to 0.98 and unique haplotypes were found in most populations.
5 Parsimony and statistical parsimony analyses of these haplotypes support the hypothesis that the introduction of *D. valens* to China was recent and originated from the Pacific North-west of the U.S.A.
6 In addition, the high haplotype diversity also suggests a large or multiple introductions. However, based on the genetics of the beetle's reproductive behaviour, this diversity may also be explained by a limited number of individuals or introductions.

Keywords Economic pest, invasive species, phylogeography, population genetics, Scolytidae.

Introduction

Inadvertent introduction of non-native insect pests through international trade is a serious threat to native ecosystems and national economies. Of these, bark beetles (Coleoptera: Scolytidae) are most often intercepted in dunnage and solid wood packing material at U.S. ports of entry (Haack, 2001). Forty-seven species of exotic scolytids are established in the U.S.A. alone, which reflects the difficulty of inspecting for insects with cryptic life cycles. These species are established within U.S. forests and urban areas and, in some cases, have greatly impacted on local economies (Haack, 2001). The introduction of U.S. bark beetle species to other countries has also occurred (Haack, 2001).

The red turpentine beetle (RTB), *Dendroctonus valens* LeConte, is a recent New World introduction to the People's Republic of China (Li *et al*., 2001; Miao *et al*., 2001). This species is native to the pine forests of North and Central America, except for south-eastern U.S.A. (Pajares & Lanier, 1990). Its entry into China is hypothesized to have taken place in conjunction with the importation of unprocessed timbers from the west coast of the U.S.A. in approximately 1980 (Sun *et al*., 2003). In 1999, an outbreak occurred in Shanxi and spread to Shaanxi, Henan and

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Hebei Provinces. The outbreak infested over 0.5 million hectares of forests of *Pinus tabuliformis* Carr and other pines and killed more than six million trees (Li *et al*., 2001; Miao *et al*., 2001). Within its native range, this species is mainly a secondary pest and epidemic populations are uncommon (Furniss & Carolin, 1977). Host volatiles are used to monitor *D. valens* populations within its native range (Rappaport *et al*., 2001). However, attraction to these semiochemical baits varies across its range; for example, populations from Wisconsin, Northern and Southern California each exhibit a strong attraction to different chemicals (Hobson *et al*., 1993; Erbilgin & Raffa, 2000). These semiochemical baits have also been tested in China. Beetles in China show a strong attraction to (+)-(3)-carene (Sun *et al*., 2004), which also elicits a strong response from a population in Southern California (N. Gillete, personal communication). In addition, *D. valens* transports fungal symbionts, which may contribute to tree mortality. These fungi also vary across the native range of *D. valens* (Owen *et al*., 1987; Klepzig *et al*., 1991; Fox *et al*., 1992).

Knowledge of the origin of the introduction could provide important insight into the ecology, and hence potential control, of the Chinese *D. valens*, as with other non-native insect pests (Kambhampati *et al*., 1991; Haymer *et al*., 1997; Scheffer & Grissell, 2003). This information could help to identify biological control agents, such as entomophagous insects, parasitic nematodes or diseases that vary throughout the beetle's native range. The few morphological differences among individuals are not consistent with particular regions (Pajares & Lanier, 1990) and determination of the origin using these characters is impossible. Molecular variation, in conjunction with phylogeographical analysis of individuals, provides an alternative means of identifying source populations (Avise, 2000; Scheffer & Grissell, 2003). Phylogeographical analysis is dependent on phylogenetic resolution, which is often difficult to obtain among individuals or populations (DeSalle & Vogler, 1994). In the case of poor phylogenetic resolution, statistical parsimony networks of DNA-types are a better predictor of intraspecific relationships (Templeton *et al*., 1992). In addition, evidence for single or multiple introduction events, introduction size and infestation growth may be inferred from the amount and distribution of genetic variation (Roderick, 1996). Low genetic variation is predicted given theoretical models of founder events (Nei *et al*., 1975) and empirical evidence from documented genetic bottlenecks (Gasparich *et al*., 1997; Slade & Moritz, 1998; Scheffer & Grissell, 2003). Similarly, extensive genetic variation among the Chinese *D. valens* would suggest a multiple or a single large introduction.

The present study examined the genetic structure of native and non-native *D. valens* populations and aimed to identify the origin of the *D. valens* infestation in China. We obtained partial mitochondrial cytochrome oxidase subunit I DNA sequences (mtDNA COI) collected from 218 individuals from populations throughout its native range and within China. Phylogenetic and genetic frequency analyses of the molecular data describe the genetic architecture and the origin of the infestation.

Materials and methods

Specimens and molecular protocols

One to 10 beetles were sampled from each of 32 locations (Table 1). Live beetles were either excised from infested host trees with a knife and forceps or attracted to a funnel trap using a blend of (–)-β-pinene (+)-3-carene and (+)-α-pinene semiochemicals (RTB lures from Phero Tech, Inc., Canada) (White & Hobson, 1993). Where possible, specimens were removed from different areas of the host tree to insure that different broods were sampled. All specimens where stored in 100% ethanol until molecular analysis. Total genomic DNA was extracted from beetle thoraces, which were removed from the rest of the body, using a silica-based spin column procedure (i.e. Qiamp, Qiagen Inc., Santa Clara, California) in accordance with the manufacturer's tissue protocol. The remaining body parts were pinned and vouchered in the Texas A&M University, Department of Entomology, insect collection.

A region of approximately 535 nucleotides of mtDNA COI, at position 2479 of *Drosophila yakuba* (Clary & Wolstenhome, 1985), was amplified via the polymerase chain reaction (PCR) with primers C1-J-2441 and TL2-N-3014 (Simon *et al*., 1994) in accordance with the methods of Cognato & Sperling (2000). Each PCR reaction contained: 35 μL ddH2O, 5 μL 10 × Taq DNA polymerase buffer (Promega Corporation, Madison, Wisconsin), 4 μL 25 mM Promega $MgCl_2$, 1 μL 40 mM deoxynucleotide triphosphates (dNTPs), 2 μL of each 5 mM oligonucleotide primer, 0.2 μL of Promega Taq DNA polymerase and 1 μL of DNA template. The PCR was performed on a thermal cycler (MJ Research, Massachusetts) under the following conditions: one cycle for 3 min at 95 °C, 0.75 min at 45 °C, 1 min at 72 °C followed by 34 cycles of 0.5 min at 94 °C, 0.75 min at 45 °C, 1 min at 72 °C and a final elongation cycle of 5 min at 72 °C.

Unincorporated dNTPs and oligonucleotides were removed from PCR reactions with a Qiaquick PCR Purification Kit (Qiagen Inc.) and were directly sequenced on an ABI 377 automated sequencer subsequent to a BigDye (Applied Biosystems, Inc., Foster City, California) fluorescent chemistry reaction. Both sense and antisense strands were sequenced for all individuals.

Sequence analysis

Using either Sequence Navigator, 1.0.1 (Applied Biosystems) or Sequencher™, 4.1 (Gene Codes Corporation, Ann Arbor, Michigan), both strands of DNA were edited, which resulted in 480 bp consensus sequences. Edited sequences of unique haplotypes were deposited in GenBank (AY724544–AY724674). Alignment of individual sequences was unambiguous because of complete amino acid conservation. No nucleotide insertions or deletions were observed. Cladistic and branch support analyses of the sequence data for 218 *D. valens* individuals and outgroup species, *D. terebrans* (Olivier) and *D. rhizophagus* (LeConte), were implemented with the program PAUP* (Swofford, 1998).

Table 1 Locality data for *Dendroctonus valens* populations used in this study

Locality	Country	State/provence	Local information	Latitude/longitude
BC	Canada	British Columbia	Okanagan Falls	49°80′N, 119.5 W
Michigan	U.S.A.	Michigan	Kalamazoo County, Kellogg Experimental Forest	42°35′N, 85°35′W
WA	U.S.A.	Washington	Chelan County	47°23′N, 120°26′W
OR.Ja	U.S.A.	Oregon	Jackson County, Quartz Gulch	42°11′N, 123°97′W
OR.Oc	U.S.A.	Oregon	Ochoco NF, 25 miles north of Prineville	44°3′N, 120°83′W
OR.Sp	U.S.A.	Oregon	Walloma-Whitman NF; Spring Creek	45°19′N, 118°19′W
CA.La	U.S.A.	California	Lassen NF, Poison Lake	40°66′N, 121°2′W
CA.Sh	U.S.A.	California	Shasta County, Shingletown	40°49′N, 121°89′W
CA.Ed	U.S.A.	California	El Dorado County, Blodgett Research Forest	38°89′N, 120°65′W
CA.Ed2	U.S.A.	California	El Dorado County, Caldor	38°36′N, 120°25′W
CA.Mo	U.S.A.	California	Monterey County, Monterey	36°06′N, 121°98′W
CA.Sb	U.S.A.	California	San Bernardino County, San Bernardino Mts	34°16′N, 116°79′W
CA.Rv	U.S.A.	California	Riverside County, San Jacinto Mts, May Valley	33°72′N, 116°68′W
CA.Sd	U.S.A.	California	San Diego County, Laguna Mt. Recreational Area	32°86′N, 116°41′W
MT	U.S.A.	Montana	Missoula; Lubrech Experimental Forest	47°08′N, 113°39′W
SD	U.S.A.	South Dakota	Black Hills	44°42′N, 103°71′W
NV	U.S.A.	Nevada	White Pine County, Little Antelope Summit	39°24′N, 115°28′W
UT	U.S.A.	Utah	Daggett County, Ashley NF	40°53′N, 109°34′W
NM	U.S.A.	New Mexico	Otero County, Cloudcroft	32°96′N, 105°74′W
Chihuahua	Mexico	Chihuahua	Municipio de Madera, Cuatro vientos	29°11′N, 108°25′W
Durango	Mexico	Durango	Pueblo Nuevo	23°45′N, 105°22′W
Aguas Calientes	Mexico	Aguascalientes	Sierra Fria	22°10′N, 92°15′W
Neuvo Leon	Mexico	Neuvo Lean	Galeana	24°33′N, 99°57′W
Mexico State	Mexico	Estado de Mexico	CICyTEC-IPN	19°04′N, 98°58′W
Distrito Federal	Mexico	Distrito Federal	Delegacion Alvaro Obregon, San Andres Totoltepec	19°15′N, 99°14′W
Michoacan	Mexico	Michoacan	Pascuala	19°26′N, 102°09′W
Chiapas	Mexico	Chiapas	Lagunas de Montebello	16°06′N, 91°43′W
Guatemala	Guatamala	Zacapa	Muni. Usumatlan; Finca San Antonio el Chico	14°58′N, 89°32′W
Henan	China	Henan	Linzhou	36°38′N, 114°29′E
Hebei	China	Hebei	Site 1	37°13′N, 117°05′E
Shanxi	China	Shanxi	Yuci	37°66′N, 112°75′E
Shaanxi	China	Shaanxi	Hancheng site 4	35°28′N, 117°40′E

Cladograms were generated under a parsimony optimality criterion. A heuristic search was performed with 50 replicates of random stepwise addition and branch swapping via tree-bisection–reconnection. All other settings were default. Bootstrap proportions were determined with 10000 replicates with 'fast' heuristic search and remaining default PAUP* settings. Little resolution was found with the cladistic analysis; thus, a statistical parsimony network (Templeton *et al.*, 1992) was created with the program TCS (v. 1.13; Clement *et al.*, 2000). A limit of nine mutational steps was set for the 95% plausible set of alternative parsimony networks (Templeton *et al.*, 1992). Some reticulations within the network were broken in accordance with Templeton *et al.* (1992), otherwise ambiguous connections were left unchanged.

Nucleotide diversity (π; Nei, 1987) was calculated with Matrix 2.0 (Posada, 2001). Haplotype diversity was calculated as $h = n(1 - \sum p_i^2)/(n-1)$ where n is the number of haplotypes and p_i is the frequency of the ith haplotype (Nei, 1987).

Results

A total of 131 mtDNA COI haplotypes were found among 218 *D. valens* individuals. Jukes–Cantor corrected nucleotide difference among haplotypes ranged from 0.2 to 16%. Nucleotide diversity (π) (0.0278 ± 0.0217),corresponding to a mean pairwise nucleotide difference among individuals (0.037 ± 0.037), was higher compared with other bark beetles (Cognato *et al.*, 2003). The majority of nucleotide change occurred at third codon positions (54%) followed by the first (16%) and second (12%) positions. Cladistic analysis of these individuals revealed 3000 parsimonious trees of 375 steps and a strict consensus of these trees was mostly unresolved (Fig. 1). Bootstrap values were low for most resolved clades (Fig. 1). The majority of branch lengths equaled only one nucleotide change. Long branches and large bootstrap values were observed for two clades, which contained individuals from Michigan and Durango + Chiapas + Guatamala (Fig. 1). Haplotypes that represented these individuals were segregated to the above clades and no Chinese individuals were found in these clades.

The clades containing individuals from Michigan and Durango + Chiapas + Guatamala were connected to the remaining phylogeny (Fig. 1) by greater than nine mutational steps, which is beyond the 95% plausible set of alternative parsimony networks. These clades were excluded from the statistical parsimony network analysis. For this analysis, a total of 119 haplotypes (Table 2) were

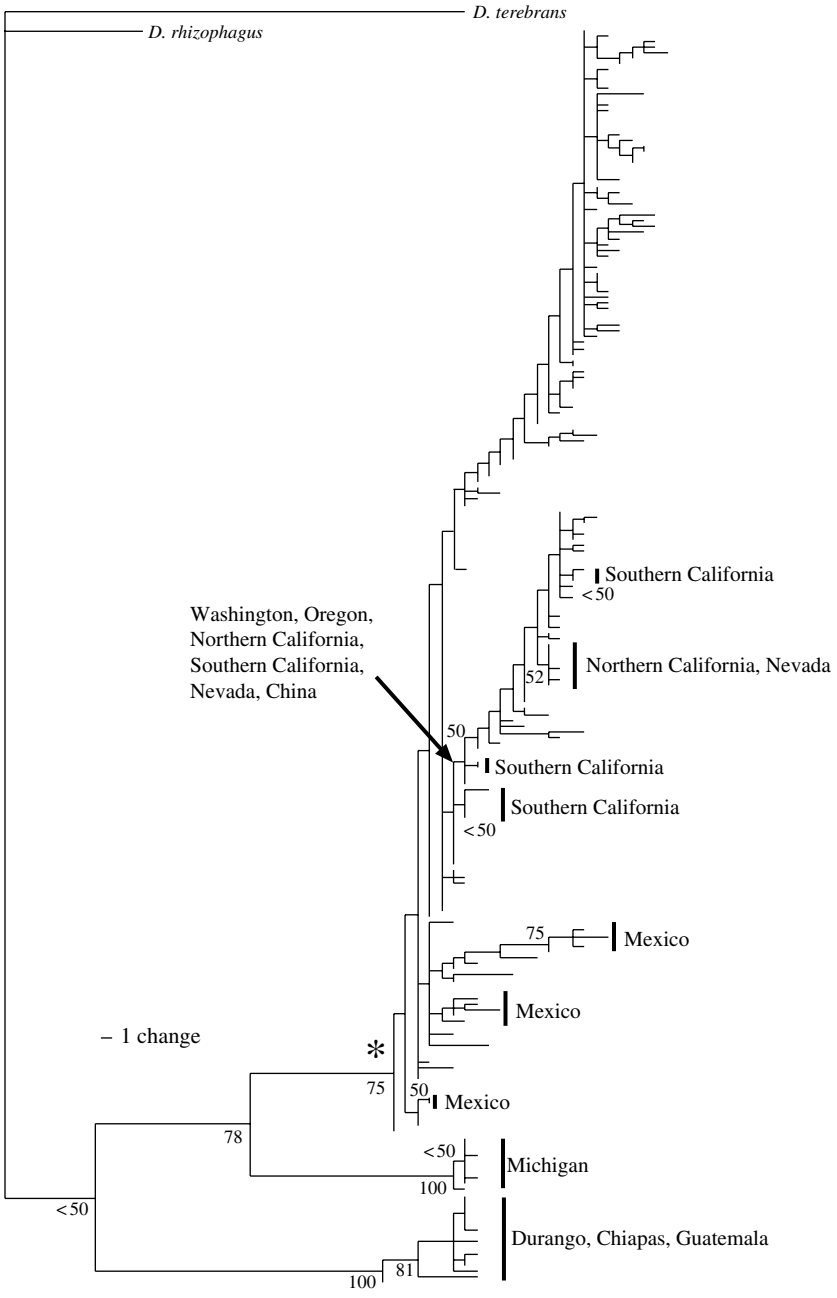

Figure 1 Phylogram of 1 of 3000 parsimonious trees found for 218 *Dendroctonus valens* individuals. Numbers indicate bootstrap proportions for clades resolved in strict consensus for the 3000 trees. Unlabelled clades were not resolved in strict consensus. Geographic origins are noted for individuals of resolved clades. *This clade contains individuals from all populations except Michigan, Durango, Chiapas and Guatemala.

found among 187 *D. valens* individuals, which equates to a high haplotype diversity ($h = 0.99$). Populations averaged 3.8 haplotypes; however, the frequency of unique haplotypes and haplotype diversity was high for most populations and at larger geographical scales (Table 2). All populations contained a unique haplotype except for one (China: Henan). Interestingly, 80% of haplotypes found in China were unique. Twenty- four common haplotypes (A to X) occurred at more then one locality and often among larger geographical regions (Table 2). For example, haplotypes B, C, E and H are widespread and all are found in China. China shares haplotypes A, D, J and M with OR.Sp, CA.Ed2 + CA.Mo, Washington and SD, respectively.

The statistical parsimony network contained multiple reticulations, which demonstrates high homoplasy among the haplotypes (Fig. 2). However, there is a general pattern of association between haplotypes and geographical locality. For example, separate clusters of Mexican and Pacific South-west (PSW) halpotypes were observed (Fig. 2). Most importantly, Chinese haplotypes were found throughout the network but grouped most often with Pacific North-west haplotypes (PNW) (Table 2, Fig. 2). These haplotypes occurred in localities

Table 2 *Dendroctonus valens* mtDNA COI haplotypes occuning at geographic regions and specific localities. Bold numbers before haplotypes indicate number of individuals with that haplotype. BC is grouped with Pacific Northwest because of its location in SW Canada. Chiapas is grouped with Central America because of its location east of the Isthmus of Tehuantepec

Geographic region	Location	Haplotypes	No. haplotypes	No. individuals	Percent unique haplotypes	Haplotype diversity
Eastern North American						0.53
	Michigan	**7** Q5, R5, S5, T5	4	10	40	
Pacific Northwest		See below	50	68	74	0.98
	BC	E, G, C2, X3	4	4	100	
	WA	J, N, I, S, T, L2, Q2 P3, T3, V3	10	10	100	
	OR.Ja	B, F, **2** R, 04, T4	5	6	83	
	OR.Oc	**2** N, I2, K2, D3, S3, R4, X4	7	8	88	
	OR.Sp	A, **4** B, B5	3	6	50	
	CA.La	C, E, R, T, D2, H2, N4	7	7	100	
	CA.Sh	K, N, Q, **3** T, N3, Q3, W3	7	9	78	
	CA.Ed	U, W2, C3, R3, M4, P4, S4	7	7	100	
	CA.Ed2	D, I, O, T, X2, L3	6	6	100	
	CA.Mo	C, D, F, K, Y3	5	5	100	
Pacific Southwest		See below	19	25	76	0.96
	CA.Sb	S, **2** W, X, A4, D4, B4, F4, J4, Q4	9	10	90	
	CA.Rv	E, Q, **2** V, C4	4	5	80	
	CA.Sd	H, U, **3** V, X, E4, G4, H4, I4	8	10	80	
Inter-Mountain		See below	29	34	85	0.98
	MT	**4** B, Z, A2, B2	4	7	57	
	SD	H, M, E2, F2, Z2, E3, H3, O3, Z3	9	9	100	
	NV	T, M2, J2, 02, R2, Y2, I3, K3	8	8	100	
	UT	N, P2, A3, J3, M3	5	5	100	
	NM	**2** H, O, A3, G3, U3	4	5	80	
Mexico		See below	22	25	88	0.97
	Chihuahua	F3, C5, G5, J5	4	4	100	
	Durango	U5, V5, W5, X5	4	4	100	
	Aguas Calientes	Y	1	1	100	
	Neuvo Leon	L4	1	1	100	
	Mexico State	**3** P, D5, H5, I5, L5, N5, 05	7	9	78	
	Distrito Federal	C, P, K5, P5, M4, Z4	6	6	100	
	Michoacan	K4, A5, E5, F5,	4	4	100	
Central America		See below	6	12	50	0.67
	Chiapas	W5, Y5, **2** Z5, **3** A6, B6	5	8	63	
	Guatemala	**4** A6	1	4	0	
China		See below	28	35	80	0.94
	Henan	**2** A, G, L, J, M	5	6	83	
	Hebei	A, L, Y4	3	3	100	
	Shanxi	**4** A, B, C, 2 D, 2 E, G, H, J, U2, V2, U4, V4, W4	13	18	72	
	Shaanxi	**2** G, E, M, G2, S2, TI2 B3	7	8	88	

from British Columbia to Monterey, California (Table 2). Exclusive Chinese haplotypes, G and L, were related to haplotypes F & M and I, respectively (Fig. 2). These latter haplotypes were sampled from four PNW and one Inter-Mountain (IM) localities (Table 2). Two Chinese haplotypes were related to the PSW haplotypes and no Chinese individuals were related to the Mexican haplotypes.

Discussion

Evidence presented in this study supports the report by Yin (2000) that the Chinese infestation of *D. valens* originated through *Pinus ponderosa* log importations from the Pacific North-west of the U.S.A. Most Chinese individuals share haplotypes with individuals from PNW and, in most cases, the individuals are from Washington, Oregon and California (El Dorado County, Monterey County) (Table 2, Fig. 2). Haplotypes unique to China are closely related to Washington, Oregon and California (El Dorado County, Monterey County). Of the 35 Chinese individuals, only two either share a haplotype with an individual from South Dakota or are closely related to individuals from PNW and PSW (Fig. 2, haplotypes Y4 and U). These exceptions are expected given the distribution of haplotypes in the beetle's native range. Individuals among PNW, PSW and IM populations demonstrate a substantial number of shared haplotypes. Thus, it is likely that a subsample of the haplotypic variation found in these regions would be present in China.

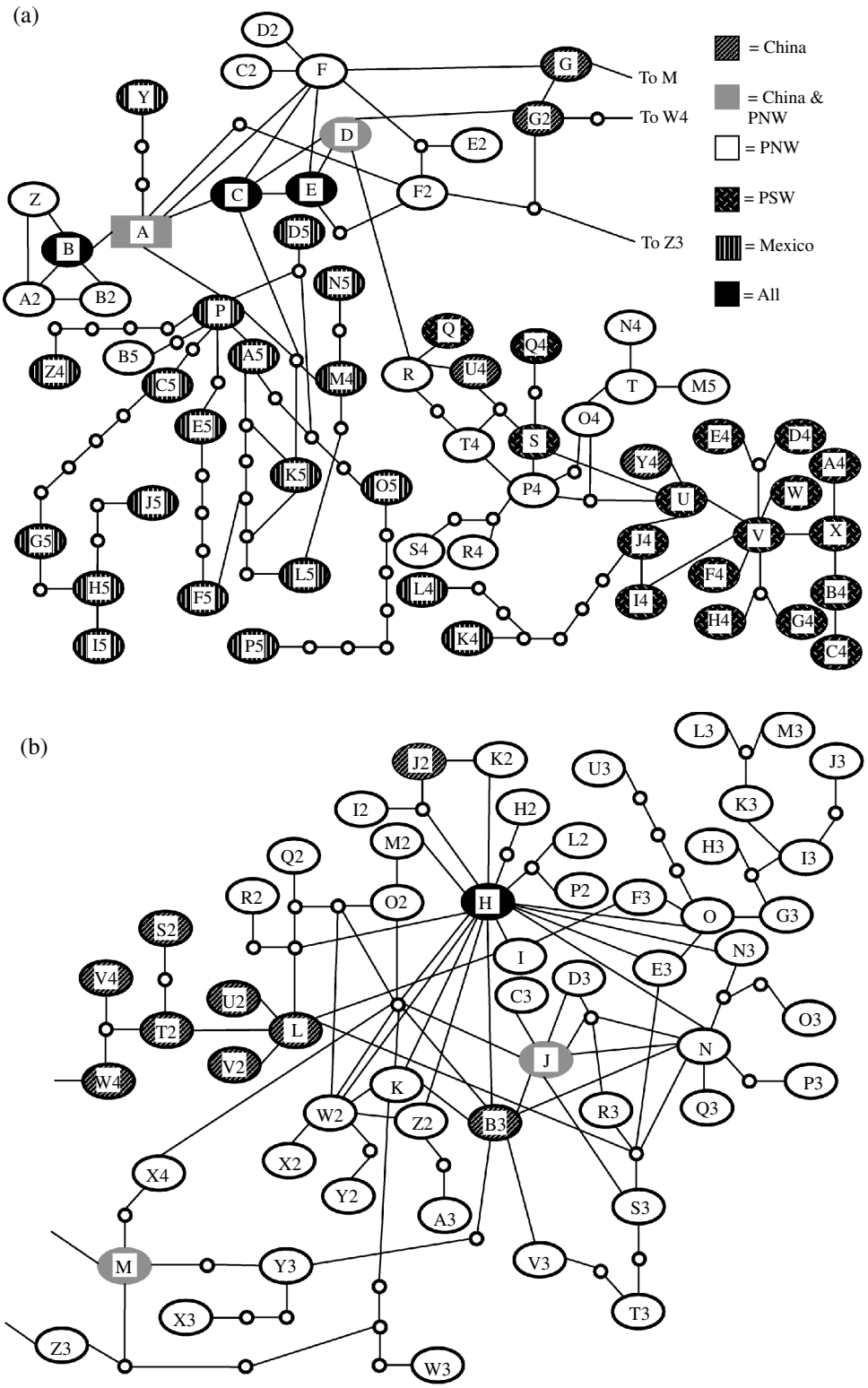

Figure 2 Parsimony network of 107 *Dendroctonus valens* haplotypes. Ovals represent haplotypes and circles represent unsampled hypothetical haplotypes. Each line segment between haplotypes and hypothetical haplotypes represents a nucleotide mutation event. The lengths of connecting lines are not significant. Oval pattern represents the area of origin for individuals. Square represents the hypothetical ancestral haplotype. Individuals with haplotype M occur in China and South Dakota.

The molecular evidence of a PNW origin is in contrast to the response of Chinese *D. valens* to (+)-(3)-carene, which suggests an affinity with *D. valens* from PSW (Sun *et al*., 2004). A lack of knowledge concerning the variation of semio-chemical response throughout the native range of *D. valens* may explain this discordance. Only the semiochemical response for Wisconsin and Northern and Southern California populations has been tested, and beetle response varied among all populations (Hobson *et al*., 1993; Erbilgin & Raffa, 2000; N. Gillette, personal communication). Thus, it is possible that other populations from, for example, Oregon, Washington or British Columbia may exhibit a strong response for (+)-(3)-carene. Whether this variation is a result of genetic or environmental (e.g. host secondary chemicals) differences is unknown.

Pinpointing the origins of invasive insects is difficult (Roderick & Navajas, 2003) and the exact origin locality of the Chinese *D. valens* is not discernible. An increased sample of *D. valens* from different PNW localities may remedy the situation. The cluster of closely-related exclusive Chinese halpotypes (G2 to L; Fig. 2) suggests a likely sampling gap within the PNW. Most likely, the incorporation of multiple nuclear loci coupled with assignment tests will further improve the identification of their origin (Davies *et al*., 1999).

The haplotypic evidence also suggests that the introduction was a recent event in terms of a geological time scale. The most divergent haplotypes found in Eastern North America, Mexico and Central America, exhibit genetic distances between 6 and 15% compared with the other halpotypes (Fig. 1). These populations were separated between 2.6 and 6.5 million years ago, assuming 2.3% mitochondrial sequence divergence per million years (Brower, 1994). The Chinese haplotypes differ by no more then two mutational events and exhibit 0.2–2% sequence divergence between related PNW haplotypes (Fig. 2). Thus, it is likely that the occurrence of *D. valens* in China was not due to an ancient dispersal event (>1 million years).

The high haplotype diversity discovered in China is in contrast to an expected decrease of genetic diversity, which is often the result of founder events (Nei *et al*., 1975; Slade & Moritz, 1998). Whether or not the mitochondrial diversity reflects higher total nuclear genetic diversity is worthy of investigation. However, greater genetic diversity is expected because higher nuclear genetic variation has been observed with low mitochondrial haplotype variation for other invasive species (Villablanca *et al*., 1998). For example, one population of *Tomicus piniperda* L., a bark beetle introduced to the U.S.A., exhibited only two common European haplotypes (Ritzerow *et al*., 2004). Although this sample was limited, another study indicated high nuclear genetic diversity among U.S. populations (Carter *et al*., 1996). Conversely, the success of *D. valens* as an invasive pest may be independent of its total genetic diversity (Tsutsui *et al*., 2000).

The high haplotype diversity also suggests a large, single introduction and/or multiple introductions of *D. valens*. However, it is possible that the high haplotype diversity resulted from one small or a few introductions. For several populations (CA.Ed, CA.Rv, CA.Sd, NM, NV and UT), all beetles attacking a host were sampled. These populations had haplotype diversities greater than 0.8 (Table 2). A limited introduction was also hypothesized for *T. piniperda,* which exhibited high nuclear genetic diversity (Carter *et al*., 1996). Thus, the importation of one or a few infested logs containing even a small number mated pairs may explain the diversity of haplotypes found in China.

The danger of the importation of even one bark beetle-infested host has been exemplified in China. High genetic diversity suggests future challenges for the control of the Chinese *D. valens* epidemic. These beetles may not uniformly respond to a particular control measure and there is the potential for the development of lineages resistant to chemical and/or biological control. Only stronger international shipping regulations and subsequent compliance will prevent other potentially disastrous introductions of forest pests (Haack, 2001).

Acknowledgements

We greatly appreciate the contribution of specimens from K. Allen, J. Borden, R. Borys, L. Chong, A. Eglitis, R. Elliott, P. Flanagan, D. Goheen, R. Haack, J. Hayes, R. Magelssen, L. Merrill, T. Petrice, D. Six, A. Stock, J. Weinz and G. Zuñiga. A. D. Harlin provided helpful recommendations for many aspects of this study. N. Gillette kindly shared unpublished data. The beetle collection in China was supported by the CAS Innovation Program (KSCX1-SW-13). This study was supported in part by the USDA Forest Service, Pacific North-west Research Station via J. Hayes, The Natural Science Foundation of China (30H71403) to J.H.S., and start-up funds to the principal author, A.I.C.

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Accepted 1 November 2004