Strong Differentiation in Mitochondrial DNA of *Dendroctonus brevicomis* (Coleoptera: Scolytidae) on Different Subspecies of Ponderosa Pine

SCOTT T. KELLEY, JEFFRY B. MITTON, AND TIMOTHY D. PAINE[1]

Department of E.P.O. Biology, Campus Box 334, University of Colorado, Boulder, CO 80309–0334

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ABSTRACT Earlier phylogenetic analysis of the bark beetle genus *Dendroctonus* based on mitochondrial DNA (mtDNA) uncovered large genetic distances between 2 populations of *Dendroctonus brevicomis*, 1 in California and 1 in Colorado. The genetic distance between the 2 populations was equivalent to that observed between fully recognized sister species of *Dendroctonus* and suggested the presence of 2 cryptic species. To test this hypothesis, we sampled *D. brevicomis* from 12 populations across its range. Using polymerase chain reaction, we amplified a 1,250-bp region of the mtDNA gene cytochrome oxidase I (COX I). A restriction site survey using 8 enzymes revealed 8 fixed differences between western (California, Oregon, Idaho, and British Columbia) and eastern (Colorado, Utah, Arizona, New Mexico) populations, suggesting that *D. brevicomis* is composed of 2 cryptic species. Moreover, the distribution of these 2 groups corresponds with the distributions of *Pinus ponderosa* variety *scopulorum* and variety *ponderosa*, the principal hosts of *D. brevicomis* over the majority of its range. We suggest that these populations of *D. brevicomis* may have become reproductively isolated as a consequence of the geographic separation of the host varieties.

KEY WORDS *Dendroctonus brevicomis*, mitochondrial DNA, speciation, ponderosa pine

PHYTOPHAGOUS INSECTS COLLECTIVELY comprise nearly a quarter of recognized species on earth, and most of these specialize to some degree in their diets (Strong et al. 1984, Bernays and Chapman 1994). Moreover, Mitter et al. (1988) demonstrated that the evolution of phytophagy in insects is, in fact, correlated with greater rates of diversification, and that clades of phytophagous insects tend to be substantially more diverse than their equally aged, nonphytophagous sister groups. Taken together, these observations suggest that host plants may influence diversification and speciation of herbivorous insects.

Over the years, numerous researchers have investigated the role of host plants in the diversification and speciation of herbivorous insects. However, the precise role that host plants might play in this process still is the subject of considerable disagreement (Futuyma and Moreno 1988, Tauber and Tauber 1989, Jaenike 1990). Much of the debate has focused on the mechanisms of speciation, and, in particular, over whether sympatric speciation is a common phenomenon (Tauber and Tauber 1989, Craig et al. 1997). During sympatric speciation, populations of phytophagous insects become reproductively isolated as a consequence of using different host species. Individuals within populations adapt to alternate hosts, and subsequently, sexual selection for mating on particular hosts eventually leads to reproductive isolation in sympatry (Bush 1975).

Alternatively, reliance on particular hosts may lead to greater chances for allopatric speciation. For instance, if the distributions of the insect's host plants become fragmented, so might the insect populations that depend on those hosts. Over time, reproductive isolation may evolve between insects on alternate hosts. In this scenario, the probability of speciation increases with the specialization of the insect and the fragmentation of the host plant distribution. Genetic differentiation as a result of habitat fragmentation has been documented in several species (Wauters et al. 1994, Hall et al. 1995, Lacy and Lindenmayer 1995), and similar patterns should be observable in insect species surveyed across fragmented host populations.

In this study, we explored the influence of host use on molecular differentiation in the western pine beetle, *Dendroctonus brevicomis* Leconte. This species is a notorious pest of ponderosa pine, *Pinus ponderosa* Lawson, as well as coulter pine, *Pinus coulteri* D. Don, with a distribution ranging across most of the western United States, up into British Columbia, and down into Mexico. Previous phylogenetic work on the genus *Dendroctonus* uncovered significant genetic divergence in mitochondrial DNA (mtDNA) (8.9% divergence in COX I) between 1 population in California on Coulter pine and 1 in Colorado on ponderosa pine (Kelley and Farrell 1999). This amount of divergence was equivalent to that observed between fully recognized sister species of *Dendroctonus*, and indicated the

[1] Department of Entomology, University of California, Riverside, CA 92521.

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Table 1. Locales of *D. brevicomis* populations included in this study. Also given are the number of individuals sampled from each population and the host plant species

No.	Locale	No. sampled	Host plant
		Eastern	
1	Durango, CO	20	*Pinus ponderosa* variety *scopulorum*
2	Lincoln NF, NM	15	*Pinus ponderosa* variety *scopulorum*
3	Stafford, AZ	15	*Pinus ponderosa* variety *scopulorum*
4	Pine Valley, UT	15	*Pinus ponderosa* variety *scopulorum*
		Western	
5	Mt. Baldy, CA	20	*Pinus coulteri*
6	Mt. Palomar, CA	15	*P. coulteri*
7	Julian, CA	15	*P. ponderosa* variety *ponderosa*
8	Sonora, CA	15	*P. ponderosa* variety *ponderosa*
9	Mt. San Jacinto, CA	10	*P. ponderosa* variety *ponderosa*
10	Bend, OR	10	*P. ponderosa* variety *ponderosa*
11	Castlegar, BC	15	*P. ponderosa* variety *ponderosa*
12	Coeur d' Alene, ID	20	*P. ponderosa* variety *ponderosa*

possibility that *D. brevicomis* might be composed of cryptic species.

The purpose of this study was to test whether the differentiation between populations of *D. brevicomis* was associated with the host species, or geographic separation, or a combination of these factors. We obtained specimens of *D. brevicomis* from 12 populations across the range of the species and from the 2 different host species in California. With these samples we performed a restriction fragment-length polymorphism (RFLP) analysis with a region of mtDNA (COX I) amplified via the polymerase chain reaction (PCR) procedure, the same region that had shown differences between the populations in California and Colorado. We then asked if there were any fixed, or diagnostic, differences between populations on different hosts or between geographic areas.

Materials and Methods

Collection and Identification. With the exception of 1 population (Durango, CO), all specimens were collected using Lindgren funnel traps (Pherotech, Delta, B.C.) baited with *D. brevicomis* pheromones. The *D. brevicomis* from Durango, CO, were collected from ponderosa pine using a small axe to peel away the bark. All specimens were placed in 70–100% ethanol and shipped to the University of Colorado where the laboratory work was conducted. Collection localities and sample sizes for all the various populations are listed in Table 1. Specimens from all populations were sent to Donald Bright for identification. Voucher specimens were deposited at the University of Colorado Museum in Boulder.

DNA Extraction and PCR. Genomic DNA was isolated and purified using procedures and materials from the QIAamp Tissue Kit (QIAGEN, Chatsworth, CA). Before DNA extraction, we dried individual beetle specimens in a speed-vac set on high temperature for 30 min, which made the specimens easier to pulverize. The specimens were then placed in a 1.5-ml Eppendorf tube and crushed with a Plexiglas rod in 180 μl of the ATL buffer supplied in the tissue kit. After following kit protocols, DNA was eluted from the QIAamp spin columns with 110 μl of 10 mM Tris-HCl pH 8.0. Using 1 μl of the DNA extraction, we then amplified a 1,250-bp fragment of the mtDNA genes cytochrome oxidase I (COX I) using PCR. For all the samples, we amplified COXI using one of the universal primers designed for insects (C1-J-1718; Simon et al. 1994), and another originally designed for chrysomelid beetles (C1-N-2962; Kelley and Farrell 1998). Using 1 μl of extracted DNA, we amplified the double-stranded DNA product under the following conditions: 10 pmole of each primer, 200 μM of each dNTP, 2.0 mM $MgCl_2$, 1x buffer (Promega, Madison, WI), and 1 unit of *Taq* DNA polymerase (Promega) in a total volume of 100 ml. Thermal cycling conditions were as follows: a 95°C denaturing step for 1 min followed by a 45°C annealing for 1 min and a 72°C extension for 2 min. This series of steps was repeated 35 times and ended in an indefinite 4°C refrigeration period until the reaction was removed from the thermal cycler.

Restriction-Site Analysis. We used the mtDNA sequence from the *D. brevicomis* populations (see Kelley and Farrell 1998) to identify restriction enzymes diagnostic for the 2 divergent populations. These sequences were deposited in GenBank under accession numbers AF067999 and AF068002. Using the Sequencher 3.0 DNA alignment program (Gene Codes, Ann Arbor, MI), we identified 8 restriction enzymes that should produce diagnostic banding patterns for the 2 different sequences: Aci I, Ase I, BamHI, HaeIII, MspI, Nla III, RsaI, and SacI. Restriction digest reactions were performed in microtiter plates to save time and reduce plastic waste (the plates could be washed out and reused). For each reaction, we used 9 μl of DNA (straight from the PCR reactions) to which we added 11 μl of master mix that included 2 μl of the appropriate 10X (buffer supplied with the enzyme), 0.2 μl BSA (if required), and 0.1 μl restriction enzyme, all up to 11 μl in water. The microtiter plates were then covered with transparent tape and placed in an incubator set at 37°C for 6 h. Finally, the digested DNA bands were separated on a 2% agarose gel with 0.5 μl 20 mg/ml ethidium bromide added per 10 ml of gel. Estimates of genetic divergence (unweighted pair-group method using arithmetic averages and neighbor-joining analyses) were obtained with the program RESTSITE version 1.2 (Miller 1991).

Results and Discussion

All 8 restriction enzymes revealed diagnostic banding patterns between eastern and western populations of *D. brevicomis*, and no variation within these groups including the populations in southern California on ponderosa and Coulter pine. Unweighted pair-group method using arithmetic averages and neighbor-joining analyses showed 2 distinct groups of populations with a sequence divergence of ≈6.9%, as follows: (1) California, Idaho, Oregon, British Columbia and (2) Colorado, Utah, New Mexico, Arizona (Fig. 1). The amount of divergence observed between these 2

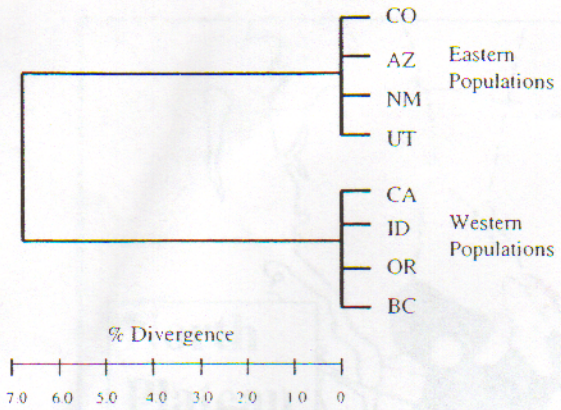

Fig. 1. Neighbor-joining analysis of genetic divergence between the various *D. brevicomis* populations sampled in this study. The analysis is based on mtDNA haplotypes (RFLPs) from the cytochrome oxidase I gene region. The 5 populations from California, 3 from ponderosa pine and 2 from Coulter pine, were lumped together in a single sample because all of the haplotypes (75) were identical by restriction fragment analysis.

groups is similar to the 8.9% divergence in the COXI sequence data found between the Colorado and California populations by Kelley and Farrell (1999).

The strong divergence in COXI observed between the eastern and western populations of *D. brevicomis* is equivalent to that found between full species of *Dendroctonus*. For example, the sister species pairs *D. pseudotsugae–D. simplex* and *D. ponderosae–D. jeffreyi* are 8.8% divergent in COXI (Kelley and Farrell 1999). The discovery of such strong genetic divergence between populations within such a well-studied insect as *D. brevicomis* comes as a surprise. However, genetic techniques have been uncovering cryptic species in extremely well-studied insects since the 1930s (Dobzhansky 1951, Soyfield et al. 1984). For example, Stock et al. (1979) discovered fixed allozyme differences between geographically separated populations of another well-studied *Dendroctonus*, *D. pseudotsugae*, indicating that other species of *Dendroctonus* also may harbor cryptic species. In fact, Hopkins (1909) originally categorized specimens from the eastern part of *D. brevicomis*'s range (down into Mexico) as a separate species, *Dendroctonus barberi* Hopkins, closely allied with *D. brevicomis*.

The substantial genetic divergence in mtDNA between these groups of *D. brevicomis* populations (6.9–8.9%) suggests that they have been separated, and reproductively isolated, for some time. Interestingly, the pattern of separation between these populations follows the east-west split of the *D. brevicomis*'s primary host plant, *Pinus ponderosa* (ponderosa pine). The eastern populations of *D. brevicomis* use *Pinus ponderosa* variety *scopulorum*, whereas the western populations use *Pinus ponderosa* variety *ponderosa* (Fig. 2). The fact that the geographic break between the 2 beetle populations matches the break with the 2 ponderosa pine varieties suggests that these sets of populations became isolated as a consequence of using the 2 geographically disjunct varieties. The 2 varieties are separated by the Great Basin, and they meet only in a narrow corridor in west central Montana called the Helena transition zone (Conkle and Critchfield 1988). Fossil evidence indicates that ponderosa pine did not grow in the Great Basin during the last glaciation (Thompson 1992); furthermore, *scopulorum* only reached northern New Mexico ≈10,000 yr ago (Betancourt et al. 1990, Barnosky et al. 1997). Thus, the 2 varieties have been in contact in the Helena transition zone for considerably <10,000 yr and, as a consequence, there has been relatively little opportunity for populations of *D. brevicomis* to move between the varieties (Fig. 2).

Alternatively, the substantial molecular divergence observed within *D. brevicomis* suggests that the molecular differentiation may have occurred several million years ago (based on even conservative molecular clock estimates; see Brower 1994), much earlier than the split between the ponderosa varieties. These variants then may have spread along with the ponderosa pine varieties to their present-day distributions. Indeed, it would be interesting to examine the DNA patterns of *D. brevicomis* from ponderosa pine in Mexico, where there are many potential ponderosa pine refugia (Rehfeldt et al. 1996). Regardless of the timing, however, the strong concordance of the molecular divergence with the distribution of the host varieties implies that the distribution of ponderosa pine has been very important in the divergence of *D. brevicomis*. This lends credence to the hypothesis that host plants have affected the population structure in this species by fragmenting and isolating the insect populations. However, the fact that we found no differences between populations of *D. brevicomis* collected from 2 different host species, ponderosa and Coulter pine, suggests that host plant species per se have not been as important in determining population structure in these insects. (Although rarely occurring in the same stands, the 2 species of trees are largely sympatric in southern California.) Thus, an allopatric model of divergence seems to fit the pattern of mtDNA variation in these insects better than a sympatric model.

One of the reasons that *D. brevicomis* might be relatively prone to the effects of resource use fragmentation is because of its rather specialized diet. For a comparison, we also examined patterns of genetic variation in COX I in the generalist *D. ponderosae*, which has a total of 12 host plants over its rather extensive range (Wood and Bright 1992). An RFLP survey of *D. ponderosae* populations collected from Colorado, Idaho, and California produced high estimates of gene flow and revealed no fixed differences among these populations (unpublished data). Therefore, we suggest that *D. ponderosae*'s broader diet allows for much more extensive levels of gene flow between populations and reduces the probability for the type of reproductive isolation observed in *D. brevicomis*.

The discovery that *D. brevicomis* may, in fact, be composed of 2 cryptic species has important conse-

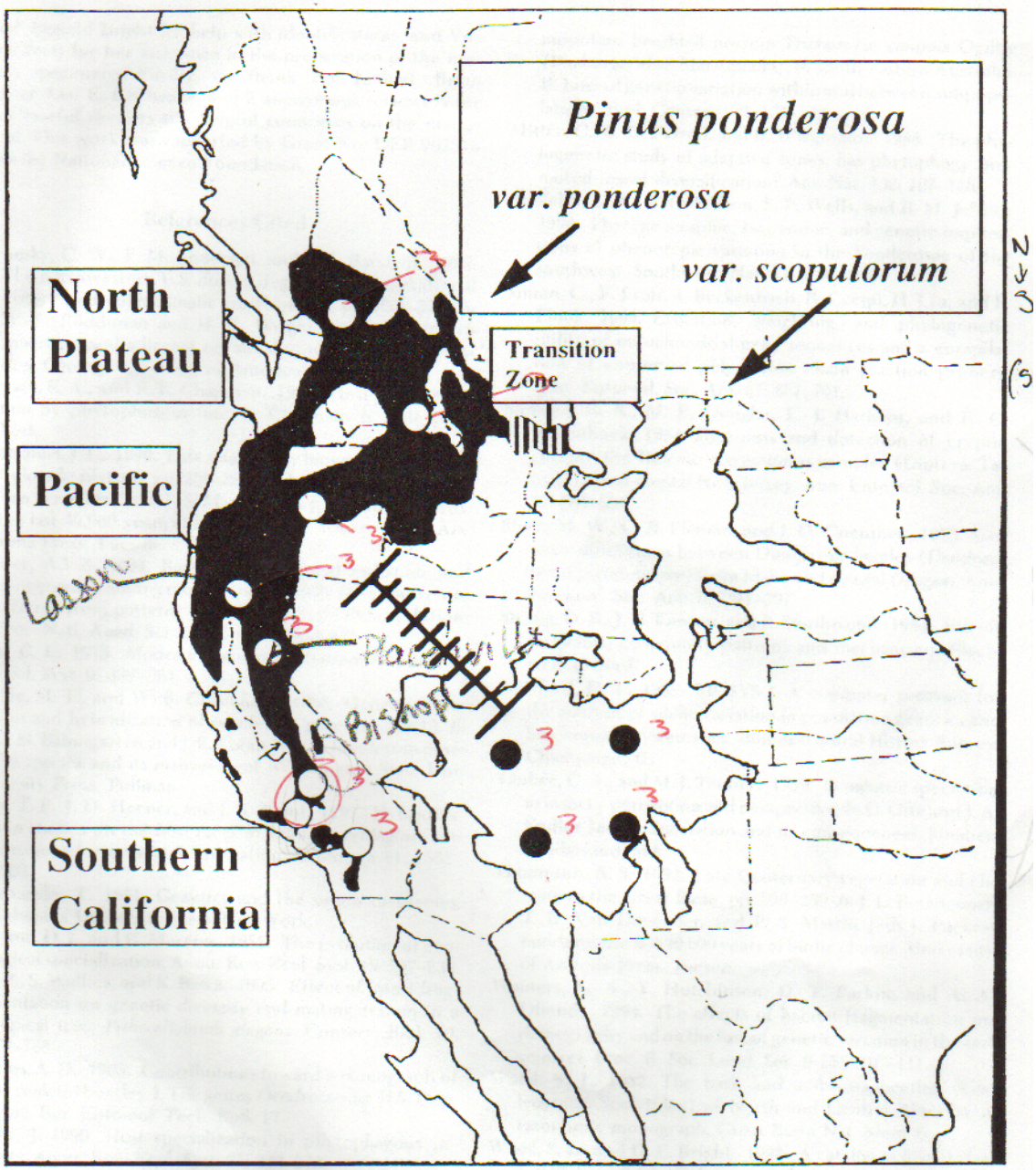

Fig. 2. Distribution of *P. ponderosa* varieties, the main host plant of *D. brevicomis*. Sampled beetle populations: ●, eastern; ○, western; black lines check off the number of fixed differences (8) between the sets of populations.

quences for the management of these insects. *D. brevicomis* is a serious pest of ponderosa pine and much effort has been spent on trying to control outbreaks of this species (Wood 1982). If the eastern and western forms of this species are as distinct in their life history traits as they are in mtDNA, they may require more specific management programs to address these differences. Clearly, more work needs to be done to find distinguishable morphological traits that could be used to separate and identify the potential cryptic species, or subspecies, within *D. brevicomis*, and to search for behavioral or life history differences that may exist between these groups.

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