Version 1 - December 2004

Identification of Coneworms in the Pacific Northwest:

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A guide to diagnostic morphological, molecular and behavioural characters of *Dioryctria* species.

Foreword

This report is intended to improve identification of coneworm moths (*Dioryctria)* in the Pacific Northwest from northern California to British Columbia. Many conifer seed orchards in this region are experiencing large *Dioryctria* infestations and improved identification is urgently needed. Control and management requires accurate identification to the species-level, and this has not been possible through traditional sources. The key and associated tutorials are intended to provide forest managers with the means to identify *Dioryctria* across this region. Morphology is promoted as the primary means for identification, with molecular and behavioral characters provided as secondary sources for species diagnosis.

A combination of morphological, molecular and behavioral traits are examined and compiled as diagnostic characters useful for identification. Key characters are presented in several formats. First, an illustrated key is provided that uses morphological characters, and is followed by detailed species diagnoses. Second, a tutorial allows the user to identify unknown specimens by comparing their DNA sequence to sequence from known *Dioryctria*. . A series of tables and figures summarize the most diagnostically important DNA bases that can potentially be used for identification in the absence of morphological characters. Finally a draft manuscript, intended for submission to Annals of the Entomological Society of America, details the methods, sampling and analyses on which this study is based.

We hope that this report will improve the identification of *Dioryctria* in the Pacific Northwest and aid in controlling these important forest pests.

Acknowledgments

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Table of Contents

Version 1 – December 2004

Morphology-based identification of *Dioryctria* species in the Pacific Northwest

Fig. 1: A: Map of areas, lines, ridges and spots important for forewing identification of *Dioryctria*; B: Map of male uncus (un) and gnathos (gn); C: Map of male valve: apical projection (ap) and accessory spine (ac); D: Map of male vesica: large cornutus (lc) and small cornuti (sc); E: Map of female genitalia: ductus bursa (db) and corpus bursa (cb).

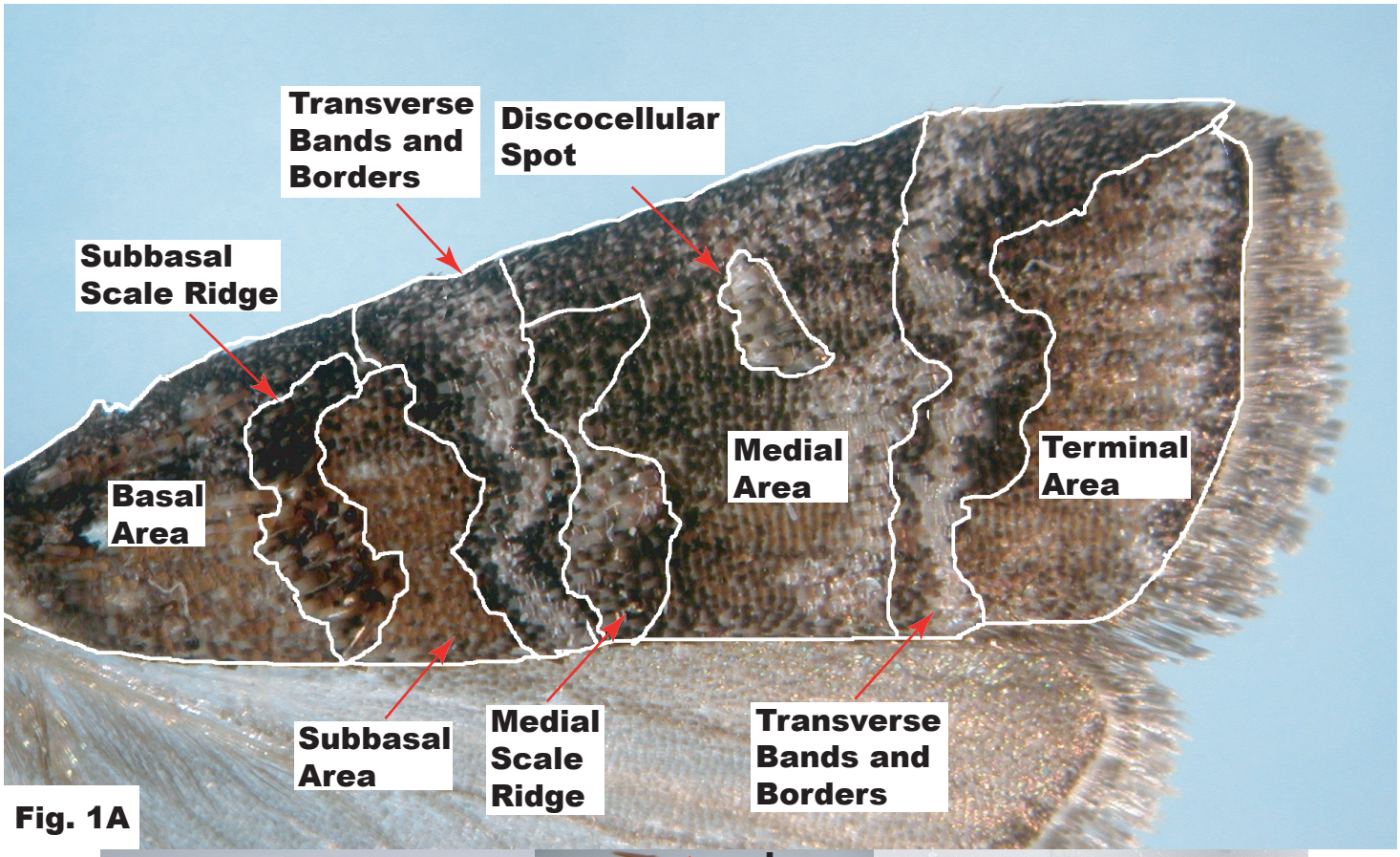


Fig. 1A

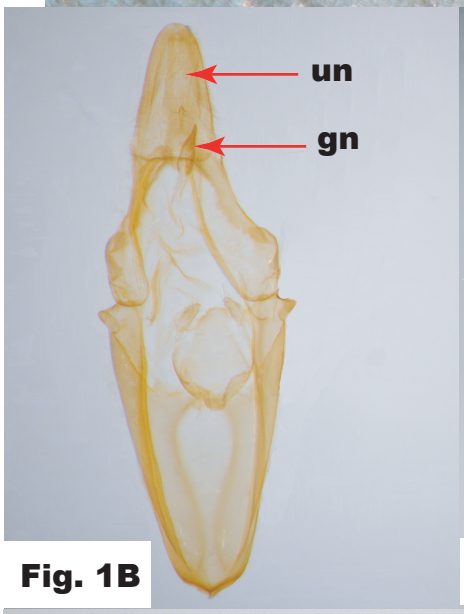


Fig. 1B

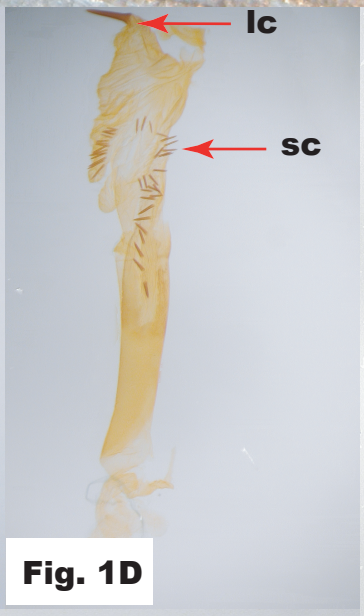


Fig. 1D

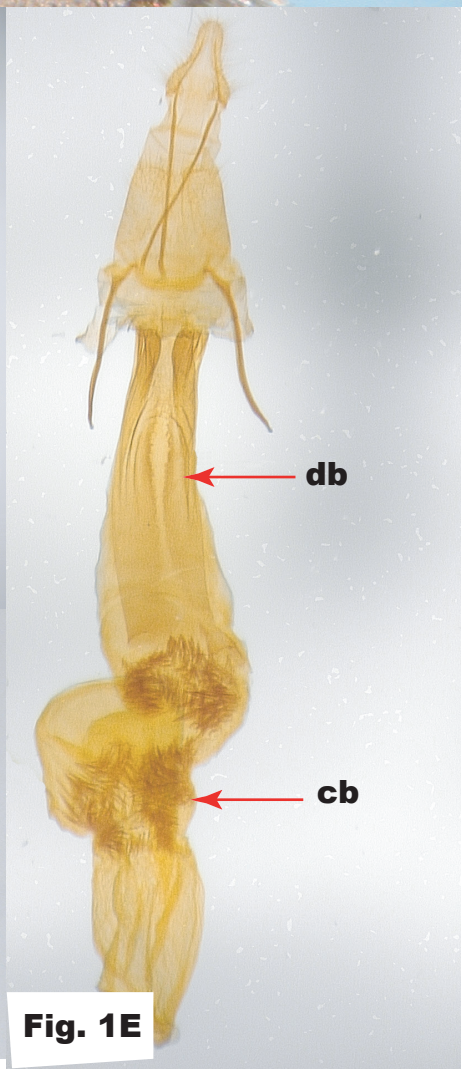


Fig. 1E

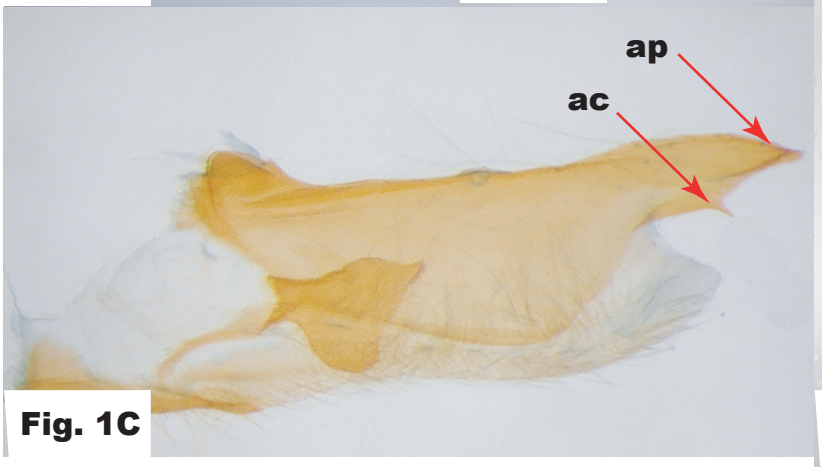
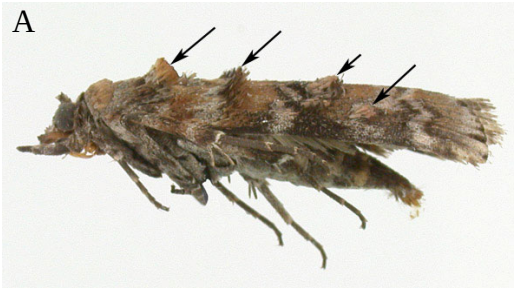


Fig. 1C

Diagnostic Key to *Dioryctria* of the Pacific Northwest

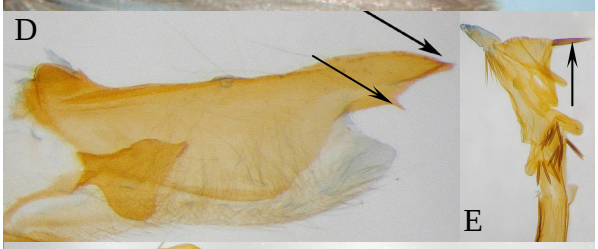
1. Forewings lacking patches of raised scales……..2

1b. Forewings with patches of raised scales (A).……4



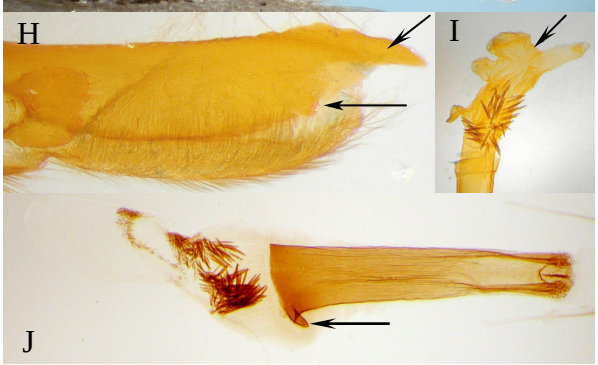
2. Forewings orange (B)……. ……..*D. aurenticella* (Grote) (Pg. 9)

2b. Forewings brown or grey (C).....3

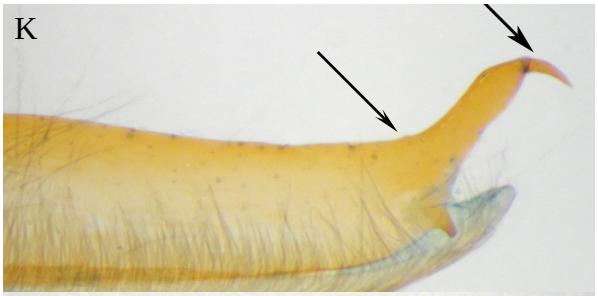


3. Forewings black and white (C); subbasal area lacking a distinct patch of orange or brown scales; valve with two distinct apical projections (D); aedeagus with large cornutus (E); ductus bursa with longitudinal membranous area (F)……. ……*D. abietivorella* (Grote) (Pg. 10)

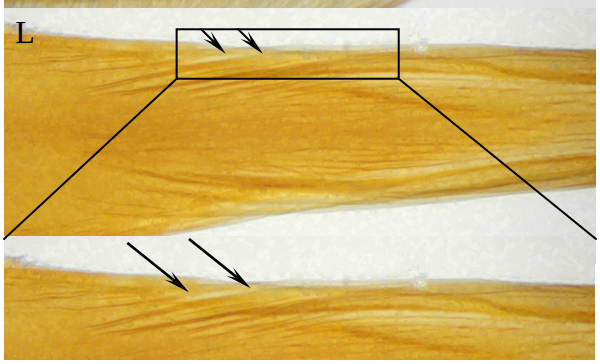
3b. Forewings brown, black and white (G); subbasal area with large distinct patch of orange or brown scales; valve with one apical projection and weak accessory spine (H), aedeagus lacking large cornutus (I); ductus bursa with recurved process on left anterior side, lacking longitudinal membranous area (J)....*D. pseudotsugella* Munroe or *D. reniculelloides* Mutuura and Munroe (Pgs. 11 & 12)

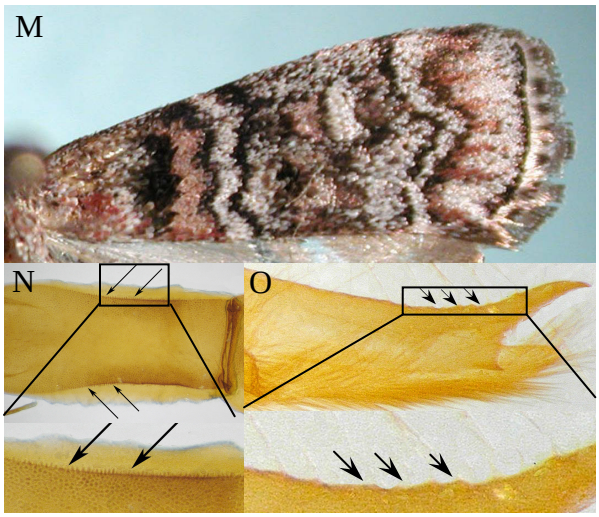


4. Forewings variable; valve with hooked apical projection, lacking dorsal tooth-like projections (K); surface of ductus bursa smooth, lacking microsculpture (L).....……5



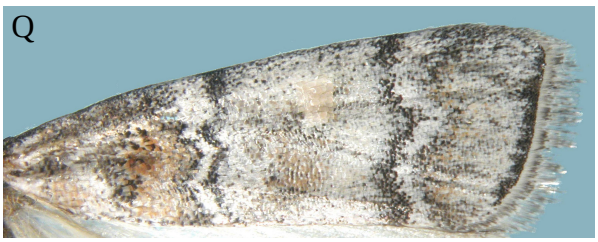
4b. Forewings with mix of black, white and red scales (M); valve with straight apical projection, dorsal surface with small tooth-like projections (N); ductus bursa with microsculputre on surface (O)... .....*D. pentictonella* Mutuura, Munroe and Ross (Pg. 13)





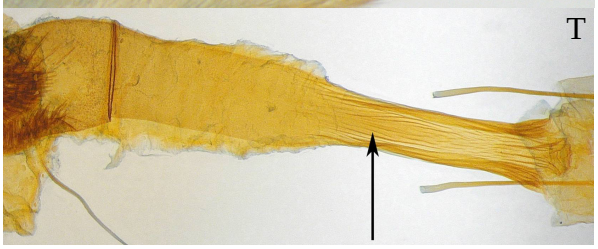
5. Forewings dark, many dark brown or black scales (P).....……...……...6

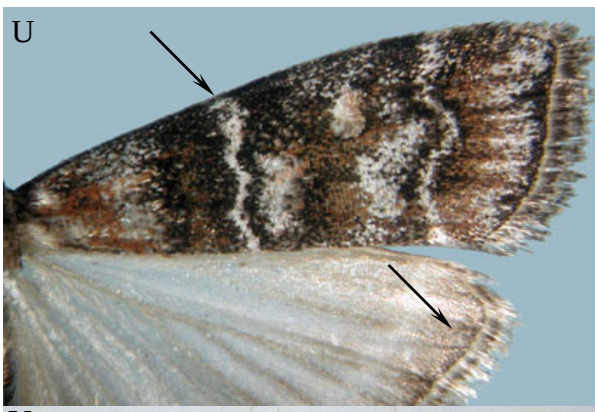
5b. Forewings pale (Q); dark brown or black scales reduced.…*D. fordi* Donahue and Neunzig (Pg. 14)

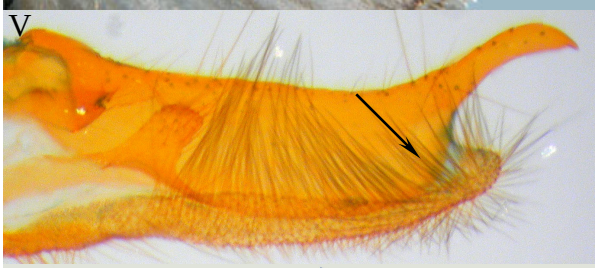


6. Transverse bands grey or brown, not distinctly white (R); hindwing dusky grey or brown; valve with prominent accessory spine along ventral edge (S); ductus bursa with anterior half broad, often folded upon itself, posterior of bursa striated with fine wrinkles (T)..... .....*D. cambiicola* (Dyar) (Pg. 15)

6b. Transverse bands distinctly white (U); hindwing pale, grey or brown scaling restricted to margin; valve lacking accessory spine along ventral edge (V); ductus bursa with heavy longitudinal wrinkles along entire length of bursa (W)..…… ….....*D. okanaganella* Mutuura, Munroe and Ross (Pg. 16)







Dioryctria Species Descriptions

Species Group: *aurenticella* Gr.

Scientific Name: *Dioryctria aurenticella* (Grote)

Common Name: Ponderosae Pine Coneworm

Habitat: Dry open forest dominated by *Pinus ponderosa*.

Seasonality: Adults fly mid-July to early August.

Identification: *Wingspan*: 10.5-14.0 mm. *Forewings*: Lacking raised scales. Primarily orange. Antemedial line and discocellular spot present. *Hindwing*: white. *Male genitalia*: Uncus lacking constriction (Fig. 3A). Valve narrow, apical projection reduced or absent and accessory spine absent (Fig. 3B). Vesica with a large cornutus and many smaller cornuti (Fig. 3C). *Female genitalia*: Ductus bursa heavily sclerotized with thin longitudinal membranous regions in corpus bursa (Fig. 3D).

Pheromone: (Z)-9-tetradecenyl acetate (3 ug) (Pasek and Dix 1989).

Life History: Larvae feed primarily in cones, though occasionally on twigs (Furniss and Carolin 1977). Feeding sites recognized by accumulation of frass around entrance hole. Pupation occurs in host.

Diet: Primary host Ponderosae pine (*Pinus ponderosa*). Also recorded on Knobcone pine (*P. attenuata*). (Mutuura and Munroe 1972; Hedlin *et al*. 1989).

Range: Restricted to western North America. Found from southern British Columbia south to California and Arizona, extending east to South Dakota and New Mexico (Neunzig 2003).

References: Mutuura and Munroe 1972; Furniss and Carolin 1977; Hedlin *et al.* 1981; Pasek and Dix 1989; Neunzig 2003

Fig. 3: *D. aurenticella*; A: Uncus round and lacking constriction; B: apical projection reduced and lacking accessory spine; C: large cornutus; D: thin longitudinal membrane

Species Group: *abietivorella* Gr.

Scientific Name: *Dioryctria abietivorella* (Grote)
Common Name: Fir Coneworm

Habitat: Coniferous forests throughout range

Seasonality: Adults fly July - September.

Identification: *Wingspan*: 10.0-13.0 mm. *Forewings:* Lacking raised scales. Primarily black and white with a prominent discocellular spot. Small beige patch in subbasal area but lacking reddish scales throughout wing. *Male genitalia*: Uncus with slight constriction.(Fig. 2A) Valve with a prominent apical projection and accessory spine (Fig. 2B). Vesica with a large cornutus and many smaller cornuti (Fig. 2C). *Female genitalia*: Sclerotized ductus bursa with a longitudinal membranous region (Fig. 2D).

Pheromone: Blend of (Z,E)-9,11-tetradecadienyl acetate (100 ug) + (Z.E)-9,12-tetradecadienyl acetate (1 ug). Additional components may be present (Grant unpublished).

Life history: Adults emerge and lay eggs under scales of new cones or under bark. Larvae will feed internally on cones, needles, twigs and under the bark of the host. Feeding sites can be recognized by the accumulation of webbing and frass around the entrance hole or around needles forming a loose shelter. Pupation occurs in host.

Diet: Recorded from a range of coniferous hosts. Fir (*Abies* spp.), spruce (*Picea* spp.) and Douglas-fir (*Pseudotsuga menzesii*) are the primary hosts. Larvae have also been recorded from various pine species (*Pinus strobus*, *P. banksiana, P. sylvestris, P. contorta*, *P. elliottii*, *P. palustris*, and *P. loblolly*). Population numbers may reach infestation levels, especially in seed orchard environments. Larvae will feed internally on cones, needles, twigs and under the bark of the host. Feeding sites can be recognized by the accumulation of webbing and frass around the entrance hole or around needles and twigs forming a loose shelter.

Range: Found transcontinentally. Recorded throughout southern Canada and south to California and North Carolina, though absent from the central plains. (Leidy and Neunzig 1986; Neunzig 2003).

References: Leidy and Neunzig 1986; Neunzig 2003

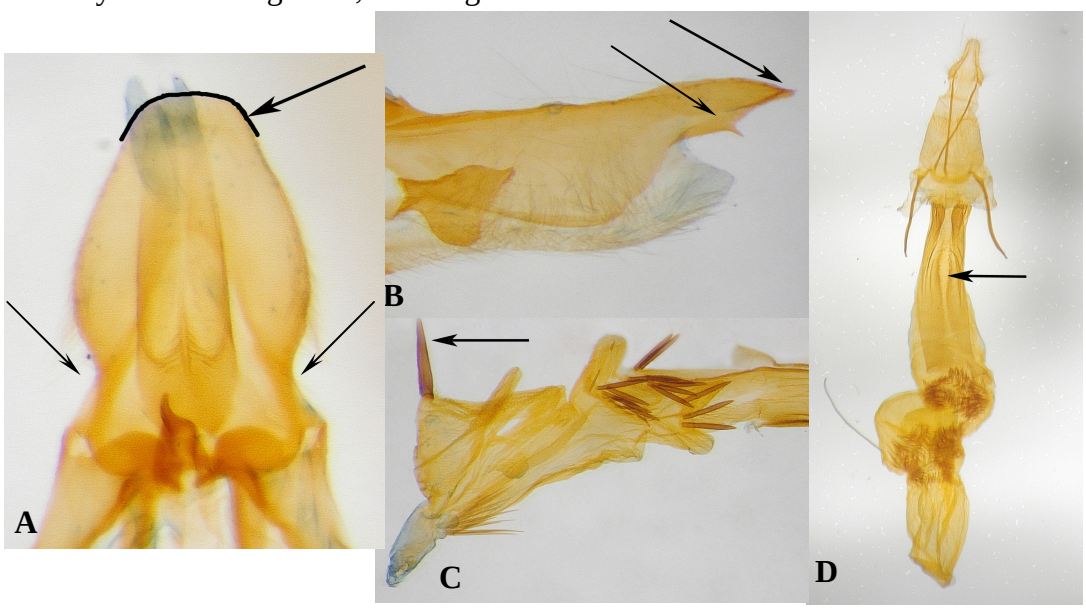


Fig 2: *D. abietivorella;* A: rounded uncus; B prominent apical projection and accessory spine; C: large cornutus; D: ductus bursa with longitudinal membrane.

Species Group: *schuetzeella* Gr.

Scientific Name: *Dioryctria pseudotsugella* Munroe

Habitat: Found in various coniferous forest types throughout range.
Seasonality: Adult fly late July – early August
Identification: *Wingspan*: 10.0-11.0 mm. *Forewings*: Lacking raised scales. Primarily brown with contrasting white transverse bands and discocellular spot. Subbasal area with pale orange-pale brown patch. Morphologically similar to *Dioryctria reniculelloides*. *Male Genitalia*: Uncus not constricted (Fig. 4A). Valve with prominent apical projection and reduced accessory spine (Fig. 4B). Vesica with many small cornuti and lacking a large cornutus (Fig. 4C). *Female Genitalia:* Ductus bursa with heavily sclerotized lateral lobe (Fig. 4D). Minor variations in transverse band dentation and coloration have been used to separate the species (Mutuura and Munroe 1973; Neunzig 2003), though intermediate specimens are present in regions of overlap. Larval host records are also used for identification though host overlap also occurs. Accurate separation of *D.pseudotsugella* and *D. reniculelloides* specimens is difficult (Sopow *et al*. 1996).
Life History: Larvae feed primarily in cones (Fig. 4E). Feeding also observed on needles and in cambium of host plants (Prentice 1965).
Diet: Primary host is Douglas fir (*Pseudotsuga menziesii)*, though larvae also found on spruce, fir and hemlock species (*Picea, Abies* and *Tsuga* spp) (Mutuura and Munroe 1973).
Range: Southern British Columbia west to Alberta and south to New Mexico.
Reference: Prentice 1965; Mutuura and Munroe 1973; Sopow 1996; Neunzig 2003

Fig. 4: *D. pseudotsugella*: A: Uncus narrows and lacks constriction; B: prominent apical projection and reduced accessory spine; C: lacking large cornutus; D: ductus bursa with sclerotized lobe; E: signs of damage to cones.

Species Group: *schuetzeella* Gr.

Fig. 5: *D. reniculelloides*

Scientific Name: *Dioryctria reniculelloides* Mutuura and Munroe
Common Name: Spruce Coneworm

Habitat: Common, found in various coniferous forest types throughout range.
Seasonality: Broad flight period depending on locality. Ranges from July – late August.
Identification: *Wingspan*: 9.5-11.0 mm. *Forewings*: Lacking raised scales. Primarily brown with contrasting white dentate bands and discocellular spot. Subbasal area with pale orange-pale brown patch. *Male genitalia*: Uncus not constricted. Valve with prominent apical projection and reduced accessory spine. Vesica with many small cornuti and lacking a large cornutus. *Female Genitalia:* Ductus bursa with heavily sclerotized lateral lobe. Genitalia images presented for *D. pseudotsugella* identical to *D. reniculelloides.* Morphologically similar to *Dioryctria pseudotsugella*. Minor variations in transverse band dentation and coloration have been used to separate the species (Mutuura and Munroe 1973; Neunzig 2003), though intermediate specimens are present in regions of overlap. Larval host records are also used for identification though host overlap occurs. Accurate separation of *D. reniculelloides* and *D.pseudotsugella* specimens is difficult (Sopow *et al*. 1996).
Pheromone: Composed primarily of (Z)-9-tetradecenyl acetate (3 ug), though two minor components (Z)-7-dodecenyl acetate (0.15 ug) and (Z)-7-dodecenal (0.15 ug) are often included (Grant *et al*. 1987).
Life History: Adults fly July – late August and lay eggs on host. Eggs hatch prior to winter and first instar larvae overwinter without feeding (Mutuura and Munroe 1973). Larvae feed primarily on cones when abundant, but switch to feeding on needles and shoots when scarce (McLeod and Daviault 1963). Larvae pupate in late June – early July.
Diet: Primary larval hosts are spruce species (*Picea* spp.), but have also been recorded on the following: Douglas fir, hemlock, fir and lodgepole pine (*Pseudotsuga menziesii, Tsuga, Abies* and *Pinus contorta*). For image of damage, see *D. pseudotsugella*.
Range: Yukon and Northwest Territories south to California and New Mexico; range extends east to Nova Scotia and New York (Mutuura and Munroe 1973).
References: Mcleod and Daviault 1963; Mutuura and Munroe 1973; Sopow *et al.* 1996; Neunzig 2003

Species Group: *baumhoferi* Gr.

Scientific Name: *Dioryctria pentictonella* Mutuura, Munroe, and Ross

Habitat: Dry, open pine forest. Preference is shown for young open stands, 1.5-10 m in height. Large populations may occur in conifer seed orchards.

Seasonality: Adults emerge late spring and fly mid-April to early June.

Identification: *Wingspan*: 9.5-12.0 mm *Forewings*: Strongly raised scales in basal area, subbasal area, medial area and discocellular spot. Primarily black and white with reddish scales in the basal, subbasal, medial and terminal areas. Forewing coloration highly variable in *D. pentictonella*, especially throughout California. *Male genitalia*: Uncus unconstricted and apically rounded (Fig. 6A). Valve with prominent apical projection and accessory spine. Small teeth present along the costal edge at the base of the apical projection (Fig. 6B). Vesica with large cornutus and many smaller cornuti (Fig. 6C). *Female genitalia*: Ductus bursa heavily sclerotized, equal width along entire length and covered with minute spicules (Fig. 6D).

Life history: Adults emerge late spring. Larvae feed on buds of host. Feeding sites identified by bundles of webbing and frass. Multiple broods often occurring in southern localities.

Diet info: Primary larval host *Pinus ponderosa*, though occasionally reared from *P. contorta* (Mutuura *et al*. 1969a)

Range: British Columbia south to California.

References: Mutuura *et al.* 1969b

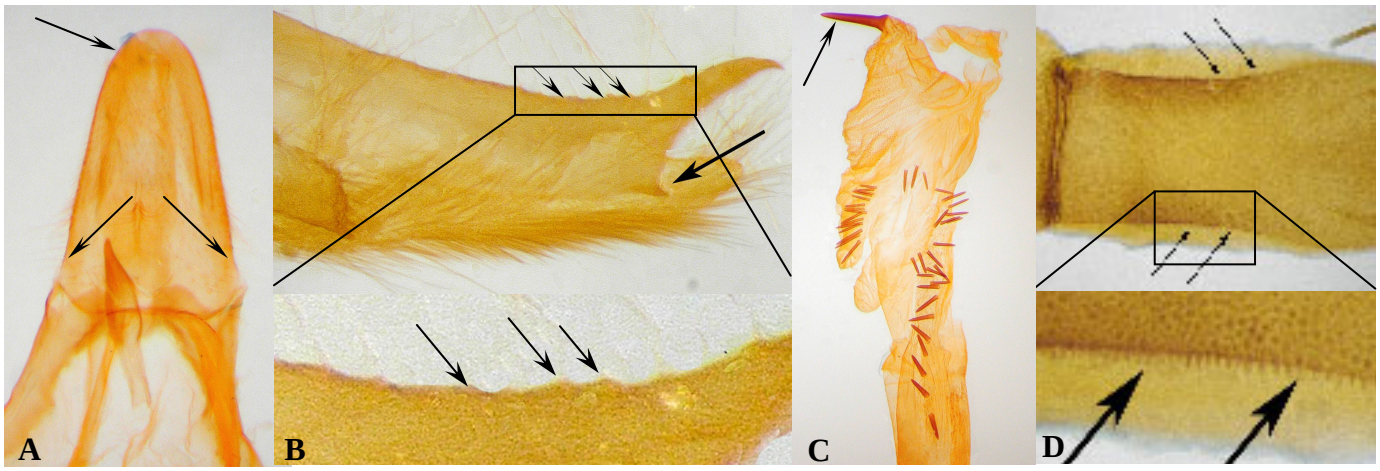


Fig. 6: *D. pentictonella*: A: uncus unconstricted and rounded; B: apical projection and accessory spine prominent, small teeth at base of projection; C: large cornutus; D: ductus bursa with minute spicules

Species Group: *zimmermani* Gr.

Scientific Name: *Dioryctria fordi* Donahue and Neunzig

Habitat: Dry woodlands, dominated by oak, pine and interspersed with grasslands.
Seasonality: Adults collected June-October, though the majority in August.
Identification: *Wingspan*: 15.0 mm. *Forewing*: with raised scales. Primarily white with pale red and grey scales. Coloration appearing overall pale and faded, lacking distinct transverse bands and reddish scaling. *Male genitalia*: Uncus constricted and expanded laterally (Fig. 9A). Valve with prominent hooked apical projection and accessory spine (Fig. 9B). Vesica with large cornutus and many smaller cornuti (Fig. 9C). *Female genitalia*: Ductus bursa elongate, with posterior region narrow and widening anteriorly. Fine longitudinal wrinkles restricted to posterior region of bursa.
Life History: Unknown
Diet: Lacking larval host records, though *Pinus sabiniana* is a possible host based on the specimen distribution (Neunzig 2003).
Range: Described from four counties in California (Kern Co., Tulare Co., San Benito Co., and San Luis Obispo Co.). Additional records include Butte Co.
References: Neunzig 2003

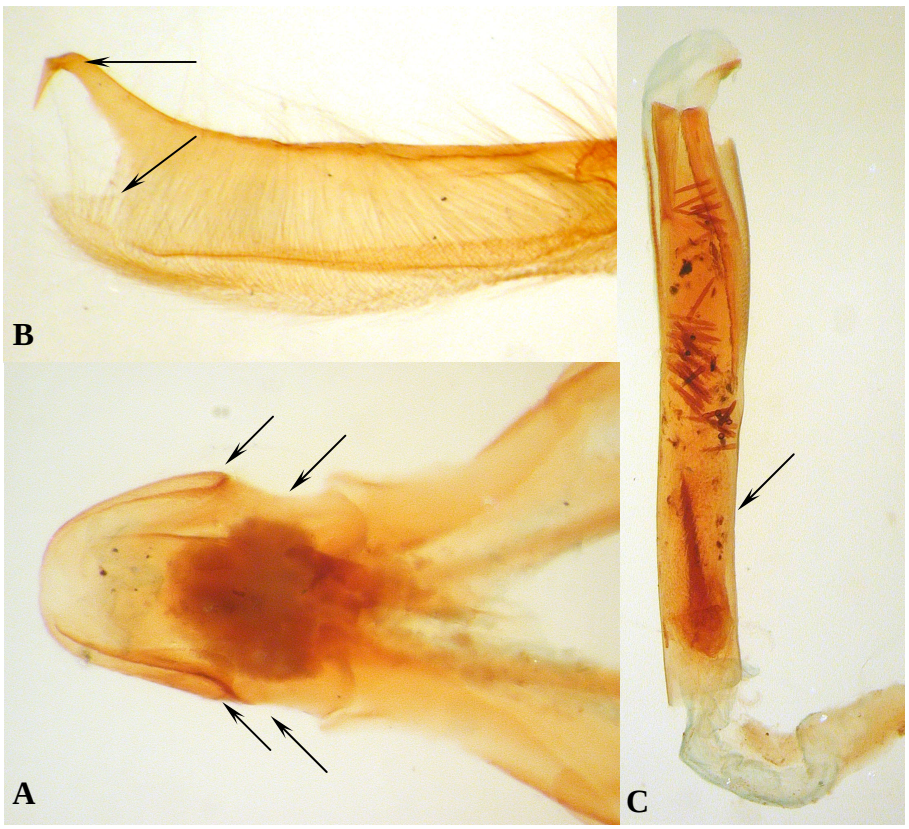


Fig. 9: *D. fordi*; A: Uncus constricted and expanded laterally; B: prominent hooked apical projection and accessory spine; C: large cornutus

Species Group: *zimmermani* Gr.

Scientific Name: *Dioryctria cambiicola* (Dyar)
Common Name: Western Pine Moth

Habitat: Dry, open forest dominated by *Pinus ponderosa* or *Pinus contorta*.
Seasonality: Adults fly from late July – late August
Identification: *Wingspan*: 13.5-15.5 mm. *Forewings:* strongly raised scales in the basal, subbasal and medial areas, as well as the discocellular spot. Predominantly reddish-brown, particularly in the costal and medial areas. *Hindwing*: distinctively dusky brown. *Male Genitalia*: Uncus constricted at base with lateral margins expanded (Fig. 8A). Valve with prominent hooked apical projection and prominent accessory spine (Fig. 8B). Vesica with large cornutus and many smaller cornuti (Fig. 8C). *Female Genitalia*: Ductus bursa elongate, with posterior region narrow and widening at anterior. Fine longitudinal wrinkles restricted to posterior region of bursa (Fig. 8D).
Life History: Larvae bore in cambium under bark, in twigs, buds and at the base of cones. Feeding sites associated with blister rust cankers on trees and are identified by large pitch masses mixed with frass at the gallery entrance (Mutuura *et al.* 1969a).
Diet: Larvae have been collected from *Pinus ponderosa* and *P. contorta*.
Range: British Columbia and Alberta south to California and New Mexico.
References: Mutuura *et al.* 1969a

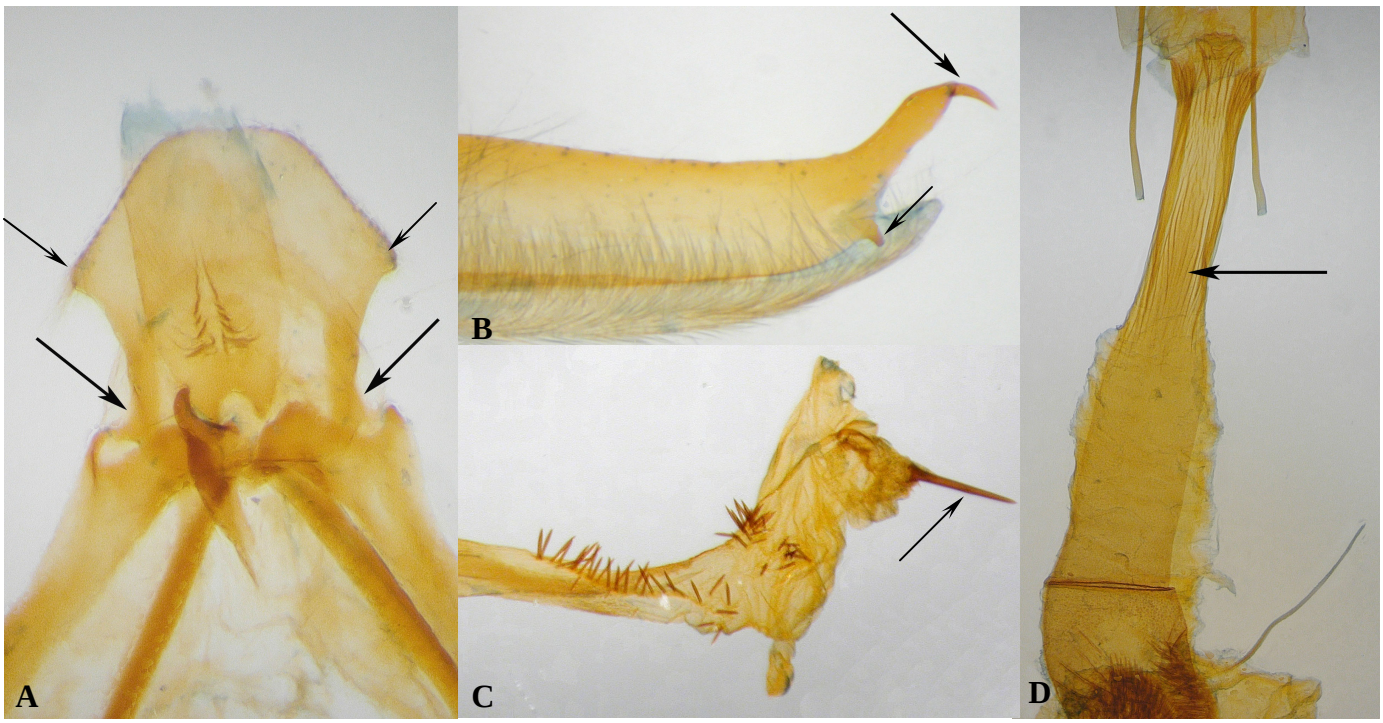


Fig 8: *D. cambiicola*: A: uncus with constricted base; B: apical projection hooked and accessory spine prominent; C: large cornutus; D: ductus bursa with narrow posterior region and fine longitudinal wrinkles.

Species Group: *ponderosae* Gr.

Scientific Name: *Dioryctria okanaganella* Mutuura, Munroe, and Ross

Habitat: Dry, open forest dominated by *Pinus ponderosa*.
Seasonality: Adults fly late June - early July
Identification: *Wingspan*: 10.0-14.5 mm. *Forewings:* strongly raised scales in basal, subbasal and medial area, as well as the discocellular spot. Primarily black and white, with extensive red scaling throughout the wing, particularly in the basal and terminal areas. *Male Genitalia*: Uncus constricted at the base (Fig. 7A). Valve with apical projection well-developed with hooked tip, lacking an accessory spine (Fig. 7B). Vesica with a large cornutus and many small cornuti (Fig. 7C). *Female Genitalia*: Ductus bursa with heavy longitudinal folds (Fig. 7D).
Life History:

Larvae feed in cambium in previous *Dioryctria* pitch masses under bark or twigs. Feeding sites are recognized by fresh pitch tubes on old pitch masses (Mutuura *et al*. 1969b).

Diet: Larvae reared from *Pinus ponderosa*.
Range: Southern British Columbia to northern California (Neunzig 2003; Roe unpublished data).
Reference: Mutuura *et al.* 1969b; Neunzig 2003

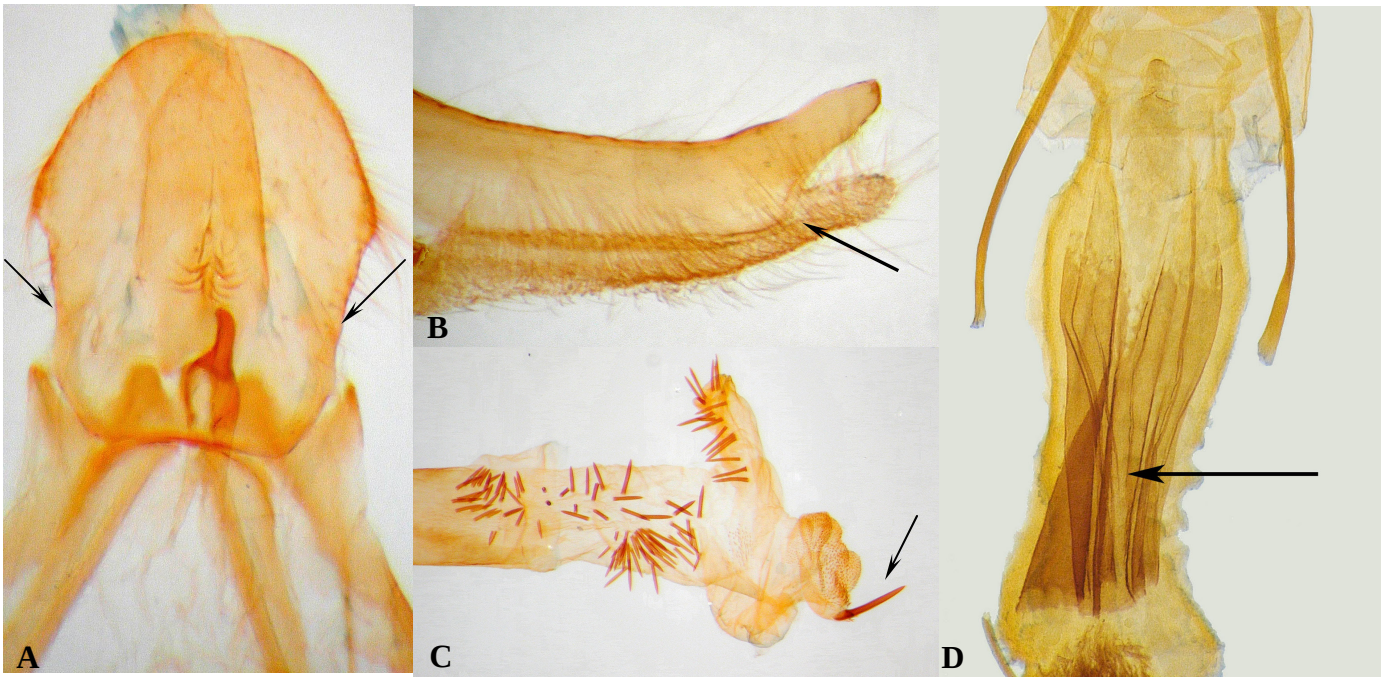


Fig 7: *D. okanaganella*; A: uncus constricted at base; B: prominent apical projection and lacking accessory spine; C: large cornutus; D: ductus bursa with heavy longitudinal folds.

Literature

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PAUP Tutorial for DNA-based Identification of *Dioryctria*

Introduction

PAUP or Phylogenetic Analysis Using Parsimony is a software program developed to aid in analyzing phylogenetic data (Swofford, 2002). Morphological characters as well as DNA sequence data for specimens or taxa can be entered in the program and their relationships are presented as phylogenetic trees. These trees can be used to compare similarity between DNA sequences as well as discover relationships among taxa.

For the purposes of this study, a how-to guide is provided that will allow the user to compare *Dioryctria* sequence data to a pre-existing data set and to aid in the identification of unknown *Dioryctria* specimens. Though sequence data is the only data analyzed in this file, results should be compared to morphological characters of each specimen to help confirm the identification.

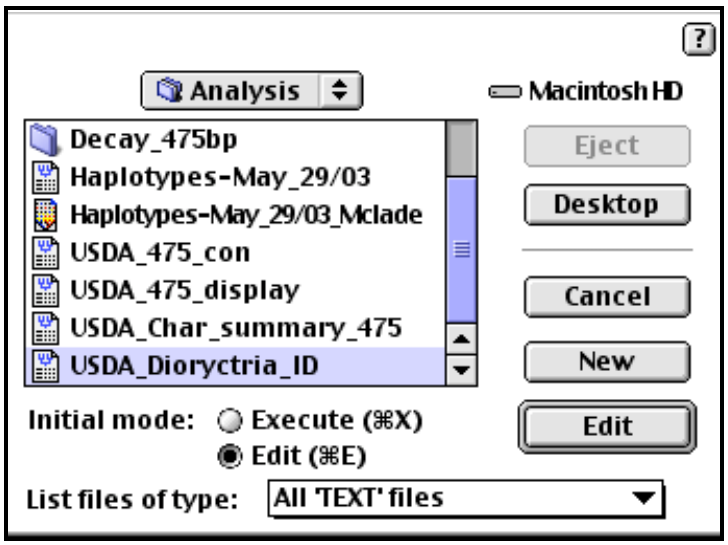
System Requirements

PAUP can run in Macintosh, Windows, Unix or DOS platforms. For detailed system requirements, see: http://paup.csit.fsu.edu/win.html

Unknown *Dioryctria* Identification

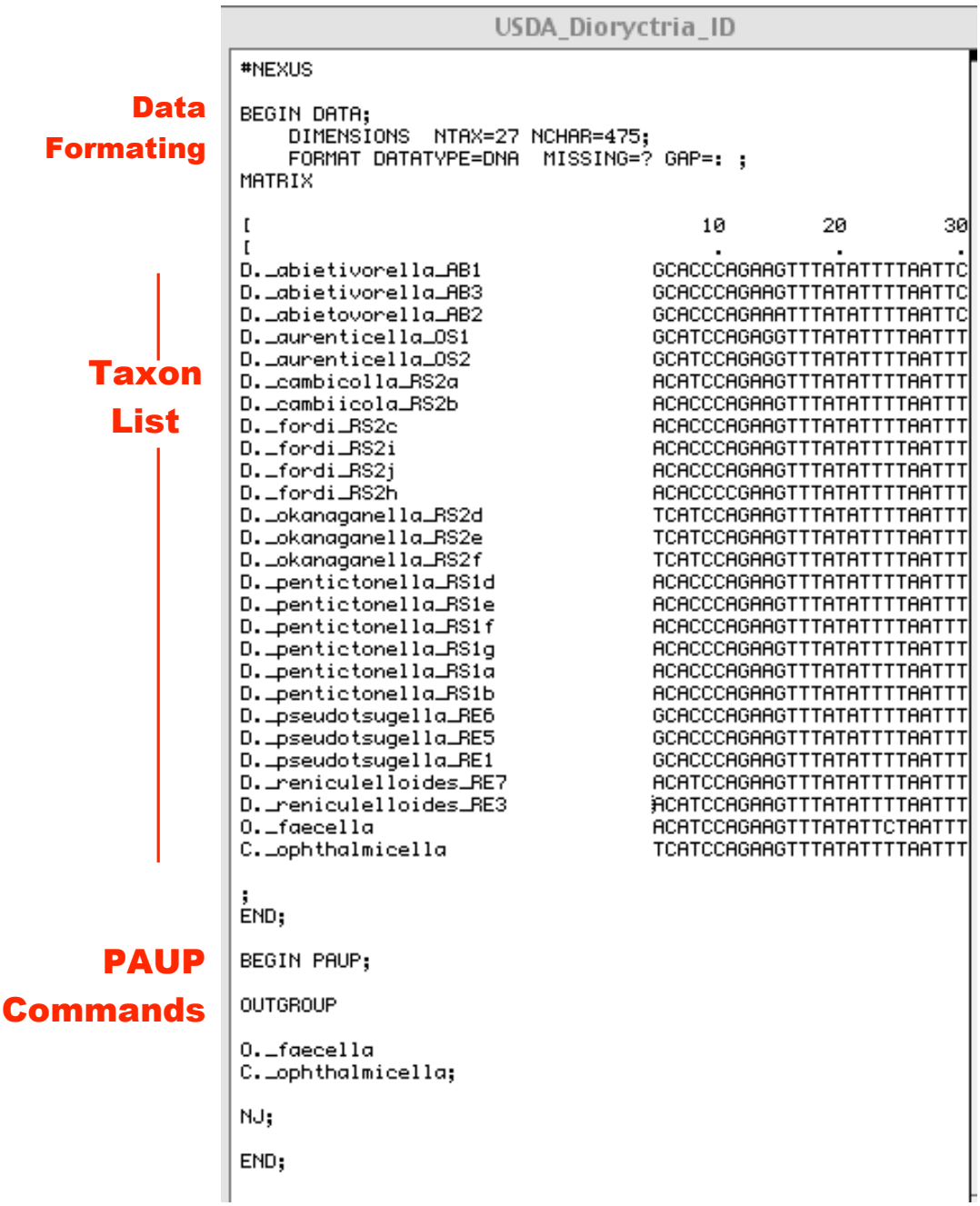
Open Dioryctria file:

- Double click PAUP icon
- Select USDA_Dioryctria_ID file from menu
- Change Initial mode: to Edit
- Click Edit



PAUP Edit Window:

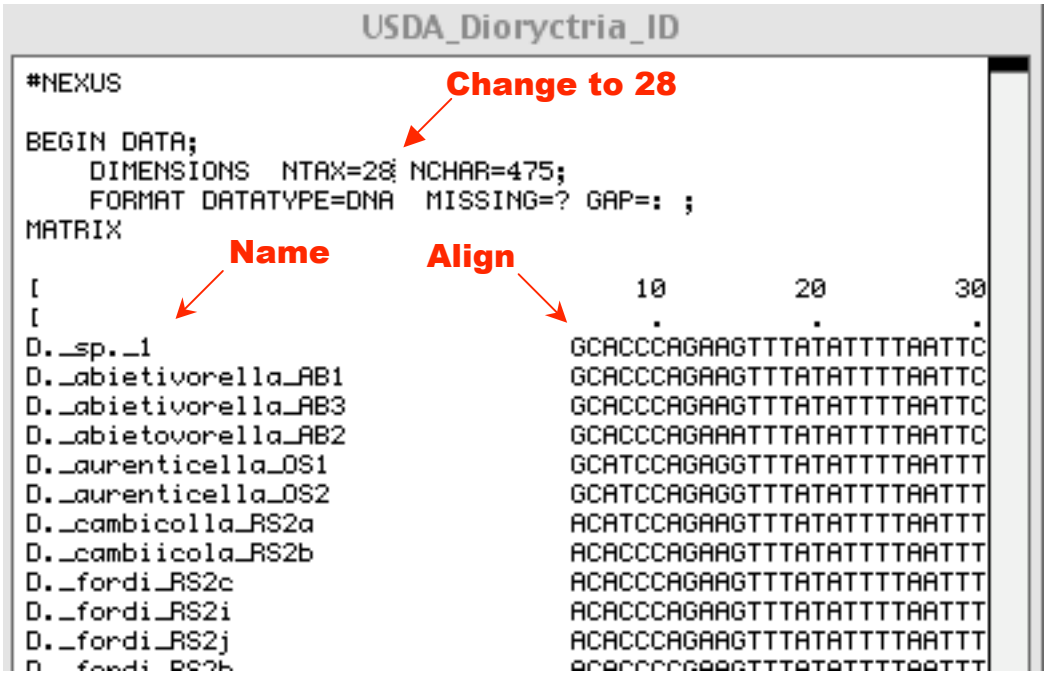
- The file will appear like this:



- You are able to insert taxa into the data matrix in this format.
- A list of commands for PAUP are found at the bottom of this window.
- This format is highly sensitive to added text. Avoid altering format or syntax of this file. Alterations may impede the functioning of the file.

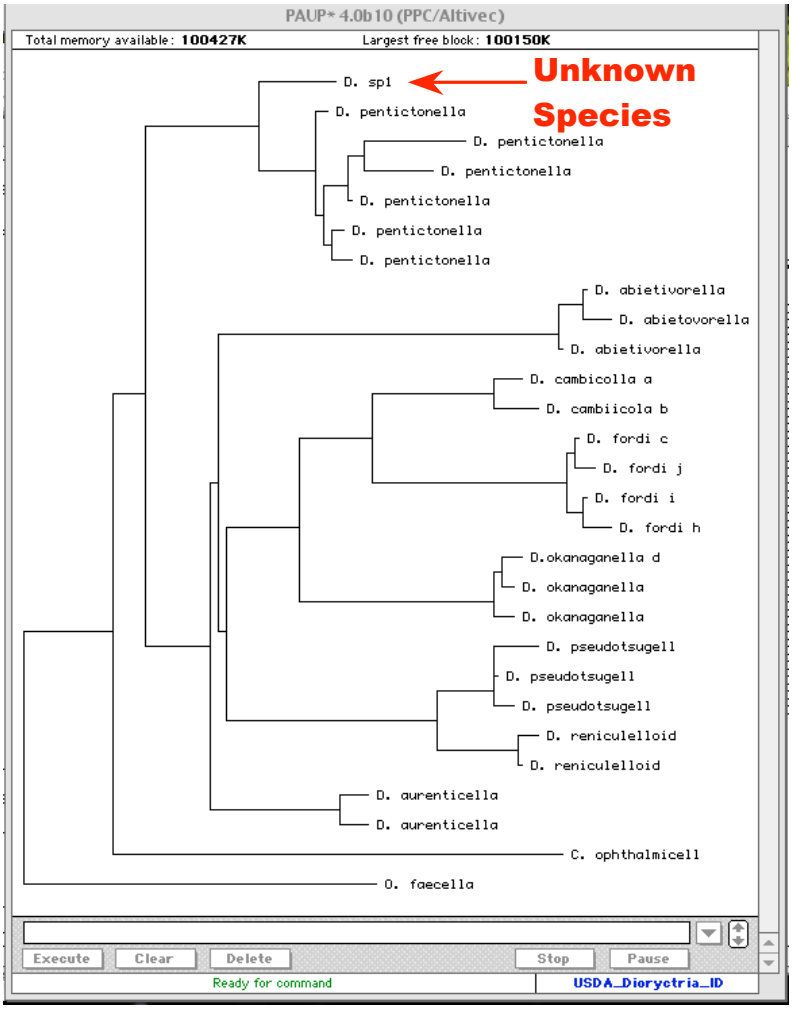
Inserting unknown Dioryctria sequence:

- Copy sequence from sequence editor or text program
- Paste in PAUP file.
- Name sequence (Do not use the following: spaces, {} [] (), ?, !, #, -, +, =)
- Align sequence with other sequence in matrix
- Adjust the NTAX to equal the number of taxa in the matrix



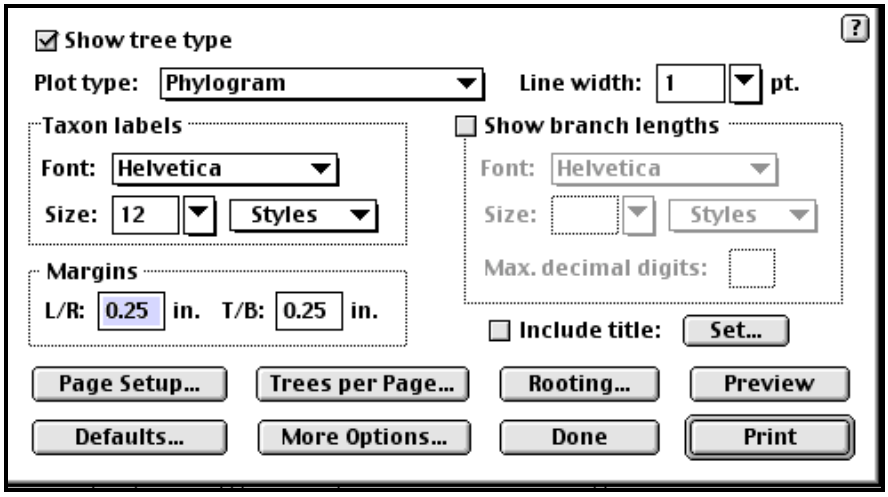
Executing PAUP file:

- Save altered file with a new file name
- Then open File: Execute….
- A neighbor-joining tree (see below) will be produced and group your sequence with the most similar *Dioryctria* sequence in the matrix.
- NOTE: if the name of the taxon is too long, it may get truncated in this window, but will be shown in the print window (see below).



- A neighbor-joining tree does not necessarily provide an accurate representation of the relationships between taxa, rather it clusters taxa according to similarity. Based on the comparison, *D. sp* 1 in the above example is most similar to *D. pentictonella*.
- It is likely that *D. sp*1 is *D. pentictonella*, but it is recommended that morphological characters should also be examined (if available) to confirm this identification.

Printing NJ tree:
- Trees: Print NJ Tree…
- The following window will open:



- You will need to change some settings to obtain a preferred view of the tree.
- Change Plot type to Phylogram (this gives you branch length information)
- You can change the type, branch thickness, and include a title if you want.
- To preview the tree prior to printing, select preview
- To print, click the Print button.

Diagnostic Character Identification
- Using the sequence and the tree that was obtained, compare the unknown sequence to the known species.
- Table 1, Figs 10 and 11 identify known diagnostic nucleotides for each species of *Dioryctria* included on the tree. Morphological characters are also provided and are outlined in the Species Diagnosis section earlier.
- Comparisons of these characters should provide accurate identification, or at least species group identification.

Literature:
Swofford, D. L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). computer program, version 4. By Swofford, D. L., Sunderland, Massachusetts.

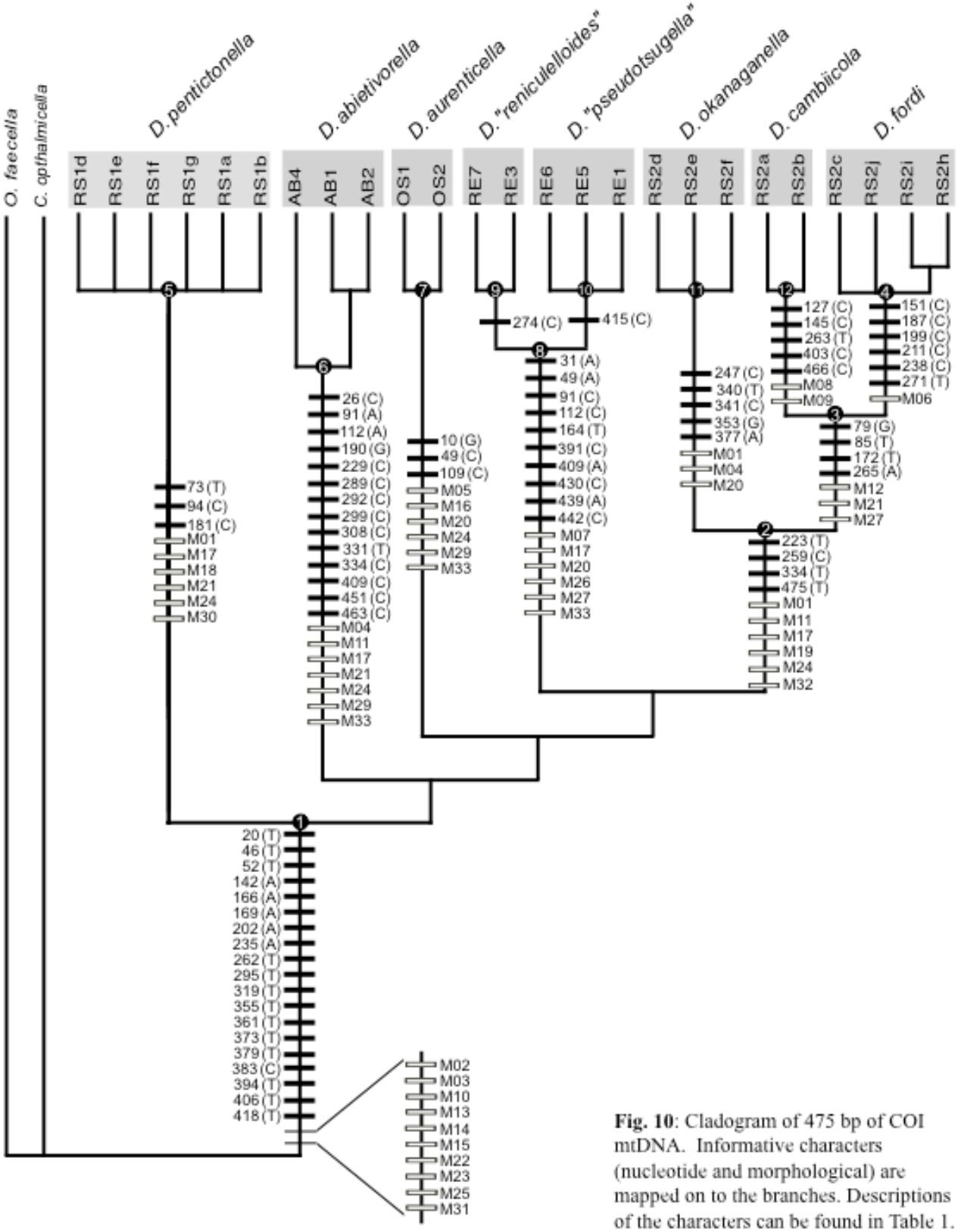


Fig. 10: Cladogram of 475 bp of COI mtDNA. Informative characters (nucleotide and morphological) are mapped on to the branches. Descriptions of the characters can be found in Table 1.

Table 1: Characters for distinguishing nodes, species groups, and lineages of *Dioryctria* found in the Pacific Northwest. For node identification see Fig. 10.

Node1: Genus Dioryctria (Zeller)

Nucleotide Characters:

Bp - *D. yakuba* #: nucleotide state
20 - 2203: T
46 - 2229: T
52 - 2235: T
142 - 2325: A
166 – 2349: A
169 – 2352: A
202 – 2385: A
235 – 2418: A
262 – 2445: T
295 – 2478: T
319 – 2502: T
355 – 2538: T
361 – 2544:T
373 – 2556: T
379 – 2562: T
383 – 2566: C
394 – 2577: T
406 – 2586:T
418 – 2601: T

Morphological Characters:

M03: FW with white discocellular spot
M02: FW with dentate transverse bands
M10: Uncus rounded and triangular
M13: Gnathos with short simple apical hook
M14: Valve broad with sclerotized costa
M15: Costa with apical projection
M22: Aedeagus sclerotized
M23: Vesica with small cornuti
M25: Ductus bursa sclerotized
M31: Corpus bursa with 1-2 groups of spines

Node 2: Raised Scales Species

Nucleotide Characters:

Bp – *D. yakuba* #: nucleotide state
223 – 2406: T
259 – 2442: C
334 – 2517: T
475 – 2658: T

Morphological Characters:

M01: Forewings with raised scales
M11: uncus constricted
M17: Prominent apical projection
M19: hooked apical spine
M24: large cornutus
M32: Larvae feed on *Pinus* sp. forming pitch masses

Node 3: zimmermani Group

Nucleotide Characters:

Bp – *D. yakuba* #: nucleotide state
79 – 2406: G
85 – 2268: T
172 – 2355: T
265 – 2448: A

Morphological Characters:

M12: uncus laterally expanded
M21: accessory spine prominent
M27: ductus bursa with fine longitudinal wrinkles

Node 4: *Dioryctria fordi* Neunzig

Nucleotide Characters:

Bp – *D. yakuba* #: nucleotide state
151 – 2334: C
187 – 2370: C
199 – 2382: C
211 – 2394: C
238 – 2421: C
317 – 2454: T

Morphological Characters:
M06: Forewing pale; primarily white scales

Node 5: *Dioryctria pentictonella* Mutuura, Munroe and Ross

Nucleotide Characters:
Bp – *D. yakuba* #: nucleotide state
73 – 2256: T
94 – 2277: C
181 – 2364: C

Morphological Characters:
M01: Forewings with raised scales
M17: Prominent apical projection
M18: apical projection with tooth-like processes
M24: large cornutus
M21: accessory spine prominent
M30: ductus bursa with minute spicules

Node 6: *Dioryctria abietivorella* (Grote)

Nucleotide Characters:
Bp – *D. yakuba* #: nucleotide state
26 – 2209: C
91 – 2274: A
112 – 2295: A
190 – 2373: G
229 – 2412: C
289 – 2472: C
292 – 2475: C
299 – 2482: C
308 – 2491: C
331 – 2514: T
334 – 2517: C
409 – 2592: C
451 – 2634: C
463 – 2646: C

Morphological Characters:
M04: FW primarily black and white – little to no red scales
M11: uncus constricted
M17: Prominent apical projection
M24: large cornutus
M21: accessory spine prominent
M29: ductus bursa with a longitudinal membranous region
M33: primarily cone feeder

Node 7: *Dioryctria aurenticella* (Grote)

Nucleotide Characters:
Bp – *D. yakuba* #: nucleotide state
10 – 2193: G
49 – 2232: C
109 – 2292: C

Morphological Characters:
M05: forewing orange/red
M16: apical projection reduced/absent
M20: accessory spine absent
M24: large cornutus present
M33: primarily cone feeder

Node 8: schuetzeella Group

Nucleotide Characters:
Bp – *D. yakuba* #: nucleotide state
31 – 2214: A
49 – 2232: A
91 – 2274: C
112 – 2295: C
164 – 2347: T
391 – 2574: C
409 – 2592: A
430 – 2613: C
439 – 2662: A
442 – 2625: C

Morphological Characters:
M07: FW primarily brown and white with pale brown subbasal patch
M17: Prominent apical projection
M20: Accessory spine reduced/absent
M26: Ductus bursa with anterior left projection

M27: Ductus bursa with fine longitudinal wrinkles
M33: Larvae primarily cone feeders

Node 9: *Dioryctria "reniculelloides"* Mutuura and Munroe

Nucleotide Characters:
Bp – *D. yakuba* #: nucleotide state
274 – 2457: C

Morphological Characters
None

Node 10: *Dioryctria "pseudotsugella"* Munroe

Nucleotide Characters:
Bp – *D. yakuba* #: nucleotide state
415 – 2598: C

Morphological Characters:
None

Node 11: *Dioryctria okanaganella* Mutuura, Munroe and Ross

Nucleotide Characters:
Bp – *D. yakuba* #: nucleotide state
247 – 2430: C
340 – 2523: T
341 – 2524: C
353 – 2536: G
377 – 2560: A

Morphological Characters
M01: FW with raised scales
M04: FW mix of black, white and red scales
M20: Accessory spine absent
M28: Ductus bursa with heavy longitudinal folds

Node 12: *Dioryctria cambiicola* (Dyar)

Nucleotide Characters:
Bp – *D. yakuba* #: nucleotide state
127 – 2310: C
145 – 2338: C
263 – 2446: T
403 – 2586: C
466 – 2649: C

Morphological Characters:
M08: FW reddish brown
M09: HW dark grey/brown

Table 2: Nucleotide variability for 475 bp of COI mtDNA across 12 selected nodes in Fig. 10. Descriptions of the morphological characters (M01-M33) are provided in Table 1.

Appendix

Taxonomy and Identification of *Dioryctria* in the Pacific Northwest (Lepidoptera: Pyralidae: Phycitinae)

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Abstract

Coneworms (Lepidoptera: Pyralidae: Phycitinae: *Dioryctria*) are important pests of conifers, particularly in seed orchard operations. Effective monitoring and control of these pests requires accurate species identification. Variable forewing morphology and a lack of diagnostic genitalic features hinder identification, prompting the search for additional characters. Mitochondrial DNA (mtDNA) sequences from the cytochrome-c oxidase I and II genes (COI and COII) were obtained from material collected at lights, pheromone traps and host plants throughout the Pacific Northwest, with additional material included from Alberta. Sampling was intensive in northern California, particularly in Chico at the Genetic Resource Center. A 475 bp fragment of COI was used to identify distinct genetic lineages and a 2.3 kb fragment of COI-COII was sequenced for a subset of specimens to increase character sampling. Eight distinct mtDNA lineages were identified. Comparisons between mtDNA variation, adult morphology, larval host association and pheromone attraction were used to identify diagnostic characters for species identification and to assign species to species groups. Species groups were well resolved and obtained from both 475 bp and 2.3 kb DNA data sets. Preliminary analyses of the 2.3 kb data set resolved several interesting phylogenetic relationships: Clade A: the genus *Dioryctria* was monophyletic, supported by both DNA and morphology; Clade F: *D. cambiicola* + *D. fordi* + *D. okanaganella*; all have similar larval feeding styles and male genitalic morphology; Clade E: Clade F + *D. pentictonella*; all have raised scales and a *Pinus* larval host. Relationships between the three lineages lacking raised scales (*D. abietivorella, D. aurenticella, D. pseudotsugella* + *D. reniculelloides*) were poorly resolved. Combination of molecular, morphological, and behavioral characters improved identification of *Dioryctria* species and provided a preliminary phylogeny of species groups.

Introduction

Dioryctria Zeller is a Holartic genus comprised of approximately seventy species, with forty species described from North America (Heinrich 1956, Munroe 1959, Mutuura *et al*. 1969a, b, Schaber and Wood 1971, Coulson *et al.* 1972, Mutuura and Munroe 1972, 1973, 1979, Mutuura 1982, Blanchard and Knudson 1983, Neunzig and Leidy 1989, Neunzig 2003). Distribution of the genus matches that of their coniferous host, with *Dioryctria* species ranging from southeastern coniferous forests to northern boreal and subarctic forests (Neunzig 2003).

The genus *Dioryctria* is one of the most easily identifiable genera in the Phycitinae (Lepidoptera: Pyralidae) (Heinrich 1956). The majority of North American species have a characteristic forewing pattern (Fig. 11), with dentate transverse bands and a white discocellular spot, though reduction of the discocellular spot occurs in at least two North American species (Heinrich 1956, Neunzig 2003). Male genitalia (Figs. 11B-D) are characterized by a rounded triangular uncus (un), a short, simple apical hook on the gnathos (gn), a broad valve with sclerotized costa (co) bearing an elongate apical projection (ap), a heavily sclerotized aedeagus with a vesica (ve) bearing many small cornuti (sc) and, often, one large cornutus (lc). Female genitalia (Fig. 11E) are distinguished by a sclerotized ductus bursa (db) and corpus bursa (cb) with one or more clusters of spines at junction of the two.

Larvae feed internally on coniferous trees, attacking regions with rapid growth (meristematic tissue) such as cones, buds, stems, cambial tissue, wounds and blister rust galls (*Endocronartium* and *Cronatrium*) (Furniss and Carolin 1977). This damage often

leads to substantial economic loss, particularly in tree farm and seed orchard operations (Lyons 1957, Blake and Wagner 1989, Mossler and Roberts 1992). Stem, trunk and shoot mining results in branch breakage and bud loss, and can cause tree deformation during heavy infestations (Hainze and Benjamin 1984). Cone feeding destroys seeds, reducing seed production, which can result in significant seed loss during years of low seed set (Schowalter *et al*. 1985).

The Genetic Resource Center (GRC) in Chico, California provides an excellent example of the damage resulting from *Dioryctria* infestations. The GRC supports 122.8 acres of grafted breeding stock and produces seedlings from three breeding zones of ponderosa pine (*Pinus ponderosa*), two breeding zones of sugar pine (*Pinus lambertiana*) and five breeding zones of Douglas-fir (*Pseudotsuga menziesii*). Unfortunately ten years of heavy *Dioryctria* infestations have drastically reduced the amount of viable seed harvested from this orchard, hindering its ability to produce seedlings for reforestation efforts in the Pacific Northwest (Koerber 1991 pub. lit.). Traditional methods of managing insect infestations, such as spraying, have been ineffective so alternative methods such as pheromone monitoring and control are being pursued.

Development of pheromone monitoring and control requires accurate species diagnosis, but forewing variability and a lack of diagnostic genitalic characters makes *Dioryctria* species identification nearly impossible. *Dioryctria* forewing patterns vary between populations and throughout the season (J. Stein, unpublished). Closely related species also lack distinctive genitalic characters compounding identification problems.

Seven species groups delineated by genitalic and forewing characters were erected (Mutuura and Munroe 1972) to improve the taxonomy and aid identification.

Even with this improved classification, identification and the taxonomy of *Dioryctria* species remains problematic (Sopow *et al*. 1996). Previous taxonomic work in North America has relied upon minor genitalic differences, forewing variability and larval host plant characters for delineating species. Unfortunately, many species live sympatrically in association with the same host plant, making it impossible to rely on these characters for species identification (Sopow *et al*. 1996). Additional characters are needed to clarify morphological and host plant variability and to reliably identify *Dioryctria* species.

Nucleotide sequence data, particularly mitochondrial DNA (mtDNA), have been useful in clarifying difficult species problems by providing a wealth of additional characters (Simon *et al*. 1994, Caterino *et al*. 2000, Sperling 2003). MtDNA is maternally inherited and essentially haploid. Mutations can accumulate rapidly, allowing the separation of closely related species. MtDNA is also robust to degradation, permitting the use of museum specimens and pheromone-collected material. The cytochrome-c oxidase regions I and II (COI, COII) have been shown to be particularly useful for delineating species problems and have been examined for many species across a range of Lepidoptera families (Caterino *et al*. 2000, Sperling 2003).

The primary objectives of this study are threefold. First, a 475 bp region of COI is used to identify distinct mtDNA lineages throughout the Pacific Northwest, from northern California to British Columbia.. Second, we relate these genetic lineages to previously described species using adult morphology, locality, larval host association and pheromone attraction. These associated characters are used to develop a key to the *Dioryctria* species in the study region, with particular emphasis on species commonly encountered in a seed orchard environment. The key focuses primarily on diagnostic

forewing and genitalic characters, relying secondarily on molecular, larval host and pheromone characters for identification. Third, we sequence 2.3 kb from each genetic lineage identified in the study region to obtain a more robust phylogeny for the species and examine relationships between species groups. Ultimately this project will form a framework for identification of *Dioryctria* species in the field, which is supported by a combination of morphology, larval host plant, pheromones, and nucleotide sequence data.

Materials and Methods

Collection Localities

A total of 179 *Dioryctria* specimens were collected throughout the Pacific Northwest (Table 3, Fig. 11) ranging from northern California to British Columbia, with two additional specimens from Alberta. Collecting was focused in conifer seed orchards, particularly at the Genetic Resource Center in Chico, CA. Samples were collected to represent the broad geographic range, pheromone attraction, larval host plant association, and morphology seen in *Dioryctria* species.

Collection Methods

Specimens were collected using a wide range of methods. Larvae were extracted as well as reared from cones, cambial tissue and pitch masses from seven conifer species (Table 3). Larvae and reared adults were preserved live in 96-100% ethanol. Adults were sampled using both light traps and pheromone lures. Light trapped specimens were frozen live at –20 ˚C or –70˚C or were placed live in 96-100% ethanol. Pheromone lures were developed and deployed by G. Grant (Grant *et al.* 1987). These lures were

developed based on previously described lures for *Dioryctria disclusa* Heinrich (Meyer *et al.* 1982), *D. abietella* (Denis and Schiffermüller) (Löfstedt *et al*. 1983), *Dioryctria clarioralis* (Walker) (Meyer *et al.* 1984), *Dioryctria amatella* (Hulst) (Meyer *et al.* 1986), *Dioryctria reniculelloides* Mutuura and Munroe (Grant *et al.* 1987), and *Dioryctria resinosella* Mutuura (Grant *et al.* 1993). Pheromone trapping has been conducted at the GRC since 1994 throughout from April – October. Pheromone blends varied based on chemical composition and concentration (Table 4). Pherocon 1CP pheromone traps (Trécé Inc., Salinas, CA) were placed in a replicated, standardized pattern in tree tops throughout the orchard to maximize trap catch (Grant *et al.* 1987). Traps were checked biweekly and specimens removed, scored for wing pattern and frozen at –20ºC. Wing pattern scoring involved categorizing specimens based on nine phenotypes previously described for the region (J. Stein, unpublished). Numbers of individuals collected and the wing phenotype present varied throughout the season. Individuals were sampled to reflect the range of variability seen within the populations and throughout the season to relate this variability to mtDNA sequence variation.

Molecular Techniques

Genomic DNA was extracted using either thoracic muscle or legs of specimens using QIAamp DNA Mini Kit (250) (Qiagen, Valencia, CA), and visualized on 0.8% agarose gels (Gibco BRL). MtDNA was amplified using a Polymerase Chain Reaction (PCR) with pairs of heterologous primers (Simon *et al*. 1994) on either a Whatman Biometra TGradient or TPersonal Thermocycler (Whatman Biometra, Göttingen, Germany) with Taq added in a hot start at the end of an initial denaturation cycle at 94ºC

for 2 min, followed by 35 cycles of 94ºC for 30 s, 45ºC for 30 s, 72ºC for 2 min, and a final extension at 72ºC for 5 mins. A 475 bp fragment in the COI region was obtained using C1-J-2183 (5' CAACATTTATTTTGATTTTTGG 3') and C1-N-2659 (5' GCTAATCCAGTGAATAATGG) for 179 individuals from populations throughout Pacific Northwest. Two haplotypes from Alberta, Canada were included to demonstrate their distinctiveness from a closely related sister lineage in the study region. Primer locations are described in Simon *et al*. (1994). From the specimens examined in the 475 bp treatment, 10 specimens were sequenced over the full 2.3 kb of COI-COII, representing seven distinct genetic lineages in the region. An additional specimen from Alberta was also sequenced for the full 2.3 kb fragment and included in the analysis for a total of 11 specimens in eight distinct lineages. A complete list of primers used to obtain the 2.3 kb fragment is included (Table 5). PCR products were cleaned using QIAquick PCR Purification Kit (250) (Qiagen, Valencia, CA) and cycle sequenced on either a Whatman Biometra TGradient or Tpersonal Thermocycler using Amersham Bioscience DYEnamic ET Dye Terminator Kit (Amersham Biosciences, Buckinghamshire, England) according to the following profile: initial denaturation at 93.0ºC for 30 s, 28 cycles of 95ºC for 20 s, 45ºC for 15 s, 60ºC for 1min, and a final extension at 60ºC for 30 s. The sequenced product was purified by filtration through Sephadex-packed columns and dried. This product was re-suspended in formamide and sequenced on an Applied Biosystems International 377 automated sequencer (Applied Biosystems, Foster City, CA). All fragments were sequenced in both directions and manually aligned to the sequence of *Drosophila yakuba* Burla (Clary and Wolstenholme 1985).

Phylogenetic Analyses

Initially the 475 bp fragments from all individuals were compared and identical mtDNA haplotypes were combined. A total of 23 unique haplotypes of *Dioryctria* were found throughout the Pacific Northwest, and Alberta specimens contributed two additional haplotypes (Table 3). Sequences were aligned by eye and nucleotides were treated as unordered, unweighted characters. Phylogenetic analysis using maximum parsimony (MP) was conducted using PAUP 4.0*b10 (Altivec) (Swofford 2002). MP analysis was performed using heuristic searches with the following parameters: 100 random addition replicates; stepwise addition; tree bisection-reconnection (TBR). Branch support was calculated using bootstrap and Bremer support values. Bootstrap values were performed with 100 bootstrap replicates using heuristic search methods as previously described. Bremer support was calculated from a strict consensus MP tree using AutoDecay 4.02' PPC. Outgroup taxa used in the analysis were *Oncocera faecella* (Zeller) (Lepidoptera: Pyralidae: Phycitinae) and *Ceroprepes ophthalmicella* (Christoph) (Lepidoptera: Pyralidae: Phycitinae).

Identification of Material

Specimens were initially identified based on previously described wing phenotypes (J. Stein, unpublished). Genitalia dissection methods were adapted from techniques outlined in Sopow *et al.* (1996) and Winter (2000). The abdomen was removed and placed in 5 ml of 10% KOH solution and boiled in a beaker of water for 10 minutes or until the abdomen was softened. The abdomen was placed in a 30% ethanol solution and scales were removed using a No. 0 insect pin and a soft hair paintbrush. The genitalia were extracted using a pair of fine forceps and a No. 0 insect pin and

disconnected from adjoining membranes. In males, vesica were everted using a capillary tube pulled to 5 μm diameter attached to microtubing and a fine syringe. This apparatus was filled with solution and then slowly ejected into the aedeagus, causing the vesica to evert. Many spines in the vesica hindered this process and evertions were aided with a hooked No. 0 insect pin. After examination, genitalia were placed in glycerin in genitalia microvials and pinned with the specimen voucher.

Images of wings and genitalia were taken with a Nikon COOLPIX 990 Digital Camera mounted on a dissecting microscope. Multiple images were taken of each specimen and compiled in AutoMontage (Syncroscopy, Frederick, MD). Wings, head capsule and remaining structures were preserved in gelatin capsules for morphological comparisons and future identifications. Vouchers are deposited in the E.H. Strickland Entomological Museum, University of Alberta, Edmonton, Alberta, Canada.

Results

Phylogenetic Analysis: 475 bp fragment

A total of 179 *Dioryctria* specimens were sequenced for a 475 bp fragment of COI. Of the 475 bp examined, 101 bp were variable and 77 bp were parsimony informative with an AT bias of 70.9% and a transition:transversion ratio of 4.73. Sequence comparisons found 25 unique haplotypes. Seventeen haplotypes were each found in at least two populations throughout the study range (Table 3), some of which were separated by large geographic distances. The remaining 7 haplotypes were unique, each found in a single individual.

A heuristic maximum parsimony search found 28 most parsimonious trees that were 175 steps in length. A representative phylogram and consensus is shown (Fig 13A & 13B). Seven genetic lineages were obtained from specimens in the Pacific Northwest, and an additional lineage was found from two Alberta specimens. These eight lineages were identified based on branch lengths, high bootstrap and Bremer support values (Fig 13A & 13B), and were composed of more than one specimen. Several lineages (Lineages 2, 3, 5) contained 2 – 3 specimens, while two lineages (Lineages 7 & 8) each contained 77 specimens. Support for each of the 8 lineages was high, with bootstrap values ranging from 79% – 100% and Bremer support values ranging from 2 – 15 (Fig. 13B).

Support for relationships between lineages was variable. Lineages 1 + 2 and Lineages 4 + 5 were well supported, though (Lineages 1 + 2) + 3 was only moderately supported by bootstrap values(Fig 13B). The monophyly of *Dioryctria* was not obtained in any of the most-parsimonious trees. Outgroup taxon *C. phthalmicella* grouped with RS1 haplotypes in all trees, though this relationship was not supported by bootstrap or Bremer support values. Multiple most-parsimonious trees resulted from rearrangements between haplotypes and low resolution of lineage relationships.

Sequence divergences within and between lineages for the 475 bp fragments were measured using uncorrected pair-wise distances. Divergence within lineages 1 to 8 ranged from 0.21% (AB1-AB2; RS2c-RS2j; RE4-RE5; RS2e-RS2f) to 1.7% (RS1d-RS1e). Divergences between lineages ranged from 1.5% (RE4-RE1) to 8.0% (AB1-RS2h). Divergences between the outgroups and the ingroup ranged from 6.7% (RS1g – *C. ophthalmicella*; RS1g – *O. faecella*; RS1b – *O. faecella*) to 9.9 % (RE5 – *C. ophthalmicella*).

Phylogenetic Analysis: 2.3 kb fragment

Based on the eight genetic lineages recovered from the 475 bp fragment, 11 representative specimens were sequenced for the entire COI-COII gene. Of the 2307 bp examined, 487 bp were variable and 297 bp were parsimony-informative with an AT bias of 73.5% and a transition:transversion ratio of 4.52.

A heuristic maximum parsimony search found a single most-parsimonious tree 782 steps in length and bootstrap and Bremer support values were calculated (Fig 13C). The eight original lineages were again resolved by the 2.3 kb data set. Most of the lineages were sequenced for using a single specimen. Where multiple specimens were sequenced (Lineages 3 & 8), the lineages were well supported with bootstrap values of 100% and Bremer support values of 42 and 62. Higher-level relationships were more resolved and showed increased support with the larger data set. *Dioryctria* was resolved as a monophyletic group and was well supported by both bootstrap and Bremer support values (Fig 13C: 94%, 14 respectively). The following relationships were also resolved and well supported: Lineages 1 + 2; (Lineages 1 + 2) + 3; ((Lineages 1 + 2) + 3) + 8; Lineages 4 + 5 (Fig. 5C).

Sequence divergences among the 2.3 kb sequences were measured using uncorrected-pairwise distances. Sequence divergence within each lineage showed less variability than the 475 bp fragment. Divergence within lineages ranged from 0.26% (RS1b – RS1d) to 0.35% (RS1b – RS1a). Divergence between lineages ranged from 1.6% to 7.4% (RE1 – RS1a). Divergence between the outgroups and ingroup ranged from 6.7% (OS1 – *O. faecella*) to 9.9% (RS2f – *C. ophthalmicella*), which was identical to the 475 bp divergences, though the species with the greatest divergence varied.

Pheromone Analysis

Eleven pheromone blends attracted *Dioryctria* species at GRC (Table 4). The numbers of individuals captured varied significantly based on lure. Lures that captured more than five individuals likely represent actual attraction and were examined in greater depth. Lures that captured singleton or doubletons were not examined further as this may represent by-catch as opposed to actual attraction. Only three pheromone lures caught more than five individuals: lure VI, VII, and VIII (Table 4). Lures VI was composed of a combination of (Z)-9-tetradecenyl acetate + (Z)-7-dodecenyl acetate in concentrations of 100 ug and 5 ug respectively. Lure VIII was also composed of (Z)-9-tetradecenyl acetate + (Z)-7-dodecenyl acetate though with lower concentrations (10 ug and 0.05 ug respectively). Lure VII was composed of two compounds, (Z,E)-9,11-tetradecadienyl acetate and (Z,E)-9,12-tetradecadienyl acetate in concentrations of 100 ug and 1 ug respectively. Weekly catch totals for these three lures were compiled for 2000 (Fig. 12). Individuals attracted to lures VI and VIII showed three peak emergences throughout the season (Fig. 12). The first peak occurred in early May, catching 25 individuals per week. The second emergence was much larger, occurred in mid-July and averaged 45 individuals per week. The final peak occurred in early October and averaged 75 individuals per week. In contrast to these two lures, individuals attracted to lure VII did not show any peaks in emergence, but were caught steadily from early May to October with an average of 5 individuals per week.

Morphological Comparisons

Forewing and genitalic characters were used to link the eight lineages to previously described species. Forewings were examined for structural characters (raised

scales), color and wing pattern. Genitalia were examined for valve structure, uncus shape, vesica spines, and ductus bursa structure. Material collected by pheromone lures were scored based on 9 forewing phenotypes delineated by J. Stein (unpublished; Type A -I) (Table 3), then examined for genitalic characters. Type A was the most numerous phenotype seen throughout the pheromone collecting period in 2000. Upon comparisons to previously identified specimens, this type corresponds to the original description for *D. pentictonella*. Types B – E, G & H showed a wide range of color variation, though were less numerous than Type A, and were also identified as *D. pentictonella*. Type F was identified as *D. aurenticella* based on the lack of raised scales and the bright orange scales on the forewing. Type F was collected once in the pheromone traps. Type I was collected in moderate numbers by the pheromone traps. This type lacked raised scales and was primarily black and white, unlike the previous types, and was identified as *D. abietivorella*. Genitalic characters were used to confirm this identification. The other specimens sampled throughout the study did not match any wing phenotypes previously described, therefore were not scored.

The morphological characters identified eight species from six species groups described from North America (Heinrich 1956; Mutuura *et al.* 1969a, b; Mutuura and Munroe 1972; Mutuura and Munroe 1973; Neunzig 2003). The lineages and their corresponding species in this study are as follows (Figs. 13C): Lineage 1: *D. fordi* (Hulst) (*zimmermani* group); Lineage 2: *D. cambiicola* (Dyar) (*zimmermani* group); Lineage 3: *D. okanaganella* Dyar (*ponderosae* group); Lineage 4: "*D. pseudotsugella"* Munroe (*schuetzeella* group); Lineage 5: "*D. reniculelloides*" Mutuura & Munroe (*schuetzeella* group); Lineage 6: *D. aurenticella* (Grote) (*aurenticella* group); Lineage 7: *D.*

abietivorella (Grote) (*abietella* group); Lineage 8: *D. pentictonella* Mutuura, Munroe & Ross (*baumhoferi* group). The specimen identified as *D. reniculelloides* was collected in Alberta and included for comparison to its sister species *D. pseudotsugella*. It should be noted that the designation of *D. reniculelloides* and *D. pseudotsugella* is based on range, rather than morphology. These two species are nearly identical morphologically, forming a species complex in this region that cannot be separated based solely on morphology at this time.

Species Diagnosis

Based on the identifications, species diagnoses are provided that outline mtDNA lineages recognized in the molecular phylogenetic analyses, species diagnoses for the study region are given. For reference to structures on wings and genitalia, see Fig. 1A-C.

abietella group
Dioryctria abietivorella (Grote)

Wing Phenotype: Type I

Figures: Forewing: Fig. 2, Genitalia: Fig 2A-D.

Diagnosis: *Forewing*: Prominently suffused with black and white scales; lacking ridges of raised scales and patches of orange/fuscous scales in subbasal area; white dentate transverse bands bounded by distinct black borders. *Hindwing*: pale buff, lacking post medial band. *Male genitalia*: Valve with pronounced apical spine, lacking hooked tip; accessory spine prominent and close to base of apical spine; uncus triangular, lacking basal constriction; vesica with large anterior cornutus and many small cornuti. *Female*

genitalia: Ductus bursa with longitudinal membranous area, equal in length to corpus bursa.

Hosts: Range of coniferous tree species. Hosts recorded from museum specimens and previous literature: Douglas-fir (*Psuedotsuga menziesii*), spruce (*Picea* sp.), fir (*Abies* sp.), pine (*Pinus* sp.). Larvae feeding primarily on cones, though also recorded on shoots, foliage, trunk and blister rust galls (*Cronartium* sp. (Uredinales: Cronartiaceae)) (Furniss and Carolin 1977).

Range: Found transcontinentally throughout range of coniferous hosts. Not known or rare from American plains states and Canadian prairies. Range extends south to Mexico. (Mutuura and Munroe 1979, Leidy and Neunzig 1986).

Notes: This species was moderately attracted to pheromone lures with either (Z,E)-9-11-tetradecadienyl acetate or its isomer (Z,E)-9-12-tetradecadienyl acetate (Lure VII, Table 3). Based on pheromone catches, a single broad flight was observed from early July to early October (Fig. 12). Collections of larvae in seed orchards throughout the region were found in all stages of development and support a single broad flight period.

baumhoferi group
Dioryctria pentictonella Mutuura, Munroe & Ross

Wing Phenotype: Type A - H

Figures: Forewing: Fig. 6; Genitalia: Fig. 6A-D.

Diagnosis: *Forewing*: Predominantly black and white scales, suffused with reddish-brown scales, though amount of black to white scales highly variable; basal area with small reddish-brown patch; subbasal area with large reddish-brown patch; medial area

with many reddish scales; distal area with dense reddish brown scales beyond outer black border of postmedial line; previous four areas highly variable between individuals and throughout range, ranging from dark reddish-brown to pale yellow, though reddish-brown phenotype most common, degree of red scaling also variable with patches shrinking to few scattered scales; four areas of strongly raised scales: small basal patch, large subbasal scale ridge, small medial patch and discocellular spot. *Hindwing*: grey, darkening at margin; postmedial band indistinct. *Male genitalia*: Valve with straight apical projection, lacking hooked tip; apical projection with small serrations along dorsal edge; accessory spine prominent on ventral edge; uncus triangular, lacking basal constriction; vesica with one large cornutus and many small cornuti. *Female genitalia*: ductus bursa fully sclerotized with minute spicules along entire surface, length equal to that of corpus bursa.

Host: Based on literature and museum records, larvae bud miners on a range of pine species (*Pinus* sp.), though most commonly found on ponderosa pine (*P. ponderosa*) (Mutuura *et al*. 1969a, Furniss and Carolin 1977, Sopow *et al*. 1996).

Range: *D. pentictonella* described from southern British Columbia, though museum material and specimens from this collection extend the range into northern California.

Notes: This species was strongly attracted to (Z)-9-tetradecenyl acetate + (Z)-dodecenyl acetate in both 100 + 5 ug and 10 + 0.05 dosages (Lures VI and VIII respectively). Additional lures caught 1 – 2 specimens of *D. pentictonella* throughout the season, though these may have been accidental. Individuals captured with these lures indicate multiple flight periods, with emergence peaks occurring in early May, June and October.

ponderosae group
Dioryctria okanaganella Dyar

Figures: Forewings: Fig. 7 Genitalia: Fig. 7A-D

Diagnosis: *Forewing*: Predominantly black and white scales; basal, medial and distal area with strong reddish-brown patches; forewings with raised scales in basal area, subbasal ridge, medial area and discocellular spot. *Male genitalia*: Valve with prominent apical spine, tip bluntly hooked; accessory spine lacking; uncus weakly constricted at base; vesica with large cornutus and many small cornuti. *Female genitalia*: ductus bursa with heavy longitudinal folds; ductus bursa slightly longer than corpus bursa.

Host: Larvae reported on old blister rust swellings or pitch masses from previous *Dioryctria* feeding on ponderosa pine (*P. ponderosa*) (Mutuura *et al.* 1969a, Furniss and Carolin 1977, Neunzig 2003).

Range: This species is described from southern British Columbia, though museum material and specimens from this study document a range extension into northern California (Mutuura *et al.* 1969a, Furniss and Carolin 1977, Neunzig 2003).

aurenticella group
Dioryctria aurenticella (Grote)

Figures: Forewing: Fig. 3; Genitalia: Fig 3A-D.

Diagnosis: *Forewing*: Primarily orange and red scales; white transverse bands moderately dentate; white discocellular spot present; white streak present in medial area extending from subbasal to medial area. *Hindwing*: buff white; lacking postmedial band. *Male genitalia*: valve with reduced apical projection; accessory spine lacking; uncus triangular, lacking basal constriction; vesica with large basal cornutus and many small

cornuti. *Female genitalia*: ductus bursa with fine medial longitudinal wrinkles and small longitudinal membranous region: ductus bursa slightly smaller than corpus bursa.

Host: Larvae recorded from cones and occasionally twigs primarily on ponderosa pine (*P. ponderosa*), but also from knobcone pine (*P. attenuata*), and limber pine (*P. flexilis*) (Mutuura and Munroe 1972, Furniss and Carolin 1977, Blake *et al*. 1989, Schoettle and Negron 2001, Neunzig 2003).

Range: Extending from southern British Columbia to Arizona and east to Montana and Colorado (Mutuura and Munroe 1972).

Notes: A single specimen was captured using a (Z)-9-tetradecenyl acetate (100 ug) lure. This lure is similar to those used by Pasek and Dix (1989).

zimmermani group
Dioryctria cambiicola (Dyar)

Figures: Forewing: Fig. 8; Genitalia: Fig. 8A-D

Diagnosis: *Forewing*: Primarily dark reddish-brown with black scales suffused throughout wing; four regions of strongly raised scales: small basal patch, large black subbasal ridge, scattered medial patch, and discocellular spot; dentate bands and discocellular spot pale brown, not distinctly white. *Hindwing*: uniformly dark brown, lacking postmedial band. *Male genitalia*: valve with prominent apical projection with hooked tip; accessory spine prominent along ventral edge of valve; uncus strongly constricted at base; vesica with large basal cornutus and many small cornuti. *Female genitalia*: ductus bursa three times longer than corpus bursa, often with fold at base, with many fine longitudinal wrinkles on posterior half of bursa.

Host: Larvae recorded on twigs, cones and wounds in a range of pine species (*P. ponderosa, P. contorta, P. coulteri*). Larvae often associated with blister rust infections and cankers (*Cronatrium* sp. and *Endocronartium* sp.) (Mutuura *et al*. 1969b, Furniss and Carolin 1977, Rocchini *et al*. 1999).

Range: Extending from southern British Columbia, south to Arizona and east to Montana and Colorado (Mutuura *et al*. 1969b, Neunzig 2003).

Dioryctria fordi (Hulst)

Figures: Forewing: Fig. 9; Genitalia: Fig 9A-C

Diagnosis: *Forewing*: Predominantly grey, strongly suffused with white, generally pale and diffused; four regions of strongly raised scales: scattered basal patch, dark subbasal scale ridge, small medial patch and discocellular spot; white dentate transverse bands indistinct, lacking dark borders; small patch of pale reddish-brown scales in subbasal and medial area; reddish-brown scales also scattered throughout basal and distal area. *Hindwing*: pale with darkening along edge; lacking postmedial band. *Male genitalia*: valve with prominent apical projection, tip strongly hooked; accessory spine prominent along ventral edge of valve; uncus strongly constricted at base; vesica with large basal cornutus and many small cornuti. *Female genitalia*: ductus bursa three times longer than corpus bursa, often with fold at base, with many fine longitudinal wrinkles on posterior half of bursa.

Host: Larvae host unknown, though based on range, *Pinus sabiniana* possible host (Neunzig 2003).

Range: Described from south central California (Neunzig 2003), though specimens from present study collected in northern California.

schuetzeella group
Dioryctria reniculelloides Mutuura and Munroe

Dioryctria pseudotsugella Munroe

Figures: Forewing: Figs. 4 & 5; Genitalia: Fig. 4A-D

Diagnosis: These two species, as mentioned previously, form a species complex throughout the study region. Based on genetic data two distinct lineages are present, but morphologically these two groups are nearly identical. Morphological identification is nearly impossible (Sopow *et al*. 1996) and separation of these two species has historically relied on larval host association and range differences. Significant overlap occurs for larval host and geographic range making in impossible to separate these putative species. An in-depth treatment with additional characters is needed to evaluate the status of these two species. If treated together, these two species can be identified from other *Dioryctria* species by the following characters: *Forewing*: Predominantly brown and white; lacking regions of raised scales; white, strongly dentate transverse bands distinct with dark brown borders; region of lighter orange/brown patch in subbasal area, though patch size variable. *Hindwing*: pale buff, darkening at margin; postmedial band pale, often broken. *Male genitalia*: Valve with prominent apical projection, lacking hooked tip; accessory spine along ventral edge reduced or absent; uncus triangular, lacking basal constriction; vesica with many small cornuti, lacking large cornutus. *Female genitalia*: ductus bursa

fully sclerotized, lacking longitudinal membranous area, sclerotized process on left anterior side, ductus bursa equal in length to corpus bursa.

Host: *D. reniculelloides*: Previous literature records adults reared from cones and shoots on a variety of spruce (*Picea* sp.), Douglas-fir (*P. menziesii*), hemlock (*Tsuga* sp.) and fir (*Abies* sp.). *D. pseudotsugella*: Recorded primarily from cones and foliage on Douglas-fir (*P. menziesii*), though also found on spruce (*Picea* sp.), hemlock (*Tsuga* sp.) and firs (*Abies* sp.). An example of feeding damage is shown in Fig. 4E.

Range: *D. reniculelloides* has been reported transcontinentally from southeastern British Columbia to the Atlantic coast and from Alaska south to California. *D. pseudotsugella* is recorded from southern British Columbia east into Alberta and south to New Mexico. Significant overlap occurs throughout British Columbia, Alberta and south through the Sierra Nevada.

Notes: The pheromone lure for *D. reniculelloides* is composed primarily of (Z)-9-tetradecenyl acetate (3 ug), though two minor components (Z)-7-dodecenyl acetate (0.15 ug) and (Z)-7-dodecenal (0.15 ug) are often included (Grant *et al.* 1987).

Discussion

MtDNA Lineages

We compared 475 bp for 179 specimens in the study region. Specimens were sampled over a range of morphological characters, pheromone lures and a wide geographic range. Twenty-five unique haplotypes were obtained and grouped into eight distinct mtDNA lineages. To help mediate the effect of a low sampling effort, we sampled a minimum of 2 specimens per lineage and included specimens from multiple

localities (Table 3: *D. cambiicola* and *"D. reniculelloides"*). A 475 bp fragment was used to broadly survey many individuals, maximizing the number of specimens sampled rather than the number of DNA characters analyzed. In the 475 bp fragments, lineages (and ultimately species) were separated by a minimum of 1.5% divergence, supported by a minimum of bootstrap of 79% and a Bremer support value of 2. The range of divergence and support values between closely related species in the same species group was high. Lineages 4 and 5 (*"D. pseudotsugella"* and *"D. reniculelloides"*) were separated by 1.5% sequence divergence, supported by moderate bootstrap and Bremer support values (79%, 2 and 93%, 2 respectively) (Fig. 13B). Conversely Lineage 2 and 1 (*D. cambiicola* and *D. fordi)* were separated by 4.0% sequence divergence, high bootstrap and Bremer supports (95%, 4 and 100%, 3 respectively) (Fig. 13B). Sequence divergence of 1.7% was seen between RS1e and the other RS1 haplotypes, but this lineage (RS1e) was not identified as distinct lineage, as it was a single specimen and lacked support. This specimen was not sequenced for the full 2.3 kb of COI-COII, though based on the degree of divergence a further examination is warranted.

Previous phylogenetic studies among Lepidoptera have demonstrated the effectiveness of using mtDNA lineages for identifying distinct species (Landry *et al*. 1999, Kruse and Sperling 2001) and delineating species boundaries (Sperling *et al*. 1999, Caterino *et al*. 2000, Sperling 2003). In species-level examinations of Lepidoptera it has been found that 2% sequence divergence generally separates sister taxa, though there have been exceptions to this standard. Exceptions to this standard are often delineated as separate species based upon morphological or behaviroural characters rather than mtDNA divergence (need lit). Generally a short fragment of COI has been used to identify these

closely related species ranging from 400 – 800 bp in length. Though this standard has been used extensively, the reliance on short COI fragments has been questioned (Wahlberg *et al*. 2003). Short fragments are often plagued by low numbers of informative characters, reducing its ability to separate closely related species. This effect is often compounded by low sampling effort in which a lineage is defined by a single specimen. Where only a single specimen is used, misleading results are often obtained and strongly supported. To avoid these problems, it is recommended to use multiple specimens for each lineage or entity and to increase the number of characters used in the analysis by increasing either fragment length or including morphological characters in the analysis (Mitchell 2000; Wahlberg *et al*. 2003; Wahlberg and Nylin 2003). Resolution of deeper phylogenetic relationships also requires increased character sampling to obtain enough phylogenetically informative characters. The lack of resolution between species groups and monophyly of *Dioryctria* suggests that increased character sampling is needed.

Though the 475 bp fragment was unable to resolve the deeper relationships between the species groups, unique sets of mtDNA characters were complied to facilitate the identification of unknown *Dioryctria* species. The morphological key and tutorial provided earlier describe the diagnostic characters that can be used for species identification and provides a means for analyzing future DNA sequences. Though mtDNA provides a reliable method for separating the species and species groups treated in this study, additional characters should be examined to confirm species identifications. MtDNA provides identifications and a phylogeny based on a single gene tree. Cases where species trees and mtDNA gene trees are incongruent are well documented,

especially between closely related species (Avis and Ball 1990, Avis 1991, Nichols 2001, Funk 2003, Ballard and Whitlock 2004). To be confident that the species delineated by the mtDNA data constitutes unique biological entities, additional characters such as morphology and larval host plant must be considered.

Morphological Identification

Morphological characteristics of specimens used in this study were examined and compared in relation to the phylogeny obtained from the mtDNA sequence data. The eight distinct lineages were supported by a suite of forewing and genitalic characters that were used to relate the lineages to previously described species (Fig. 14). The diagnostic forewing and genitalic characters were compiled into a key for morphological identification.

Though morphological characters were generally able to identify the lineages to species, wide variation (or lack thereof) caused two major difficulties. First, specimens in Lineage 8, identified as *D. pentictonella* (RS1a-f), showed a wide range of forewing variation, ranging from pale, nearly white phenotypes to dark red and black phenotypes. The most common phenotype (Type A) was similar to the original description for *D. pentictonella* (Mutuura *et al.* 1969a), but the other wing phenotypes were quite variable. The wing phenotypes described by J. Stein (unpublished) were developed with the intent of linking forewing variation to genetic variation or pheromone lure, though no correspondence to these factors was found. A single haplotype (RS1a) contained specimens with seven wing phenotypes (Type A-E, G, H) and was found attracted to seven pheromone lures (Table 4). These observations suggest that this species is highly variable with a wide range of phenotypic variability in a single population.

Phenotypic plasticity is quite common in Lepidoptera, with some cases showing wide ranges of forewing variation. Manley (1973) provides an example of extreme phenotypic forewing variation where 119 forms were described for a single species of tortricid (Tortricidae: *Acleris cristana*). A wide range of factors can cause changes in wing phenotype of genetically identical individuals, such as: temperature, photoperiod, altitude and larval host plant. Variation caused by seasonal changes in temperature and photoperiod is termed seasonal polyphenism and has been well-documented in Lepidoptera (Nijhout 1991). Seasonal morphs tend to differ in coloration, rather than pattern or shape. Often the background color and pattern elements are much darker and more pronounced early in the season, then become paler in the summer generations. Examples of seasonal polyphenism can be found in both tropical and temperate species (lit), with several well-document cases in North America (Shapiro 1976, Nijhout 1991). The pheromone lures in this study collected dark phenotypes of *D. pentictonella* early in the season, then as the season progressed the pale phenotypes became more prevalent. This trend suggests that the wide range of variation seen in *D. pentictonella* may be a form of seasonal polyphenism triggered by temperature or photoperiod, rather than genetic differences between individuals.

Difficulties also arose in the morphological delineation of Lineages 4 and 5 (RE1-RE3; RE4, RE5). These two lineages were separated by 1.5% for both the 475 bp and 2.3 kb fragment, but lacked morphological characters supporting this distinction. The specimens display a broad range of variation in forewing morphology throughout the range and genitalic characters lacked variable characters useful for distinguishing these two species. Genitalic characters placed these two lineages in the *schuetzeella* group.

Specimen collection localities were used to tentatively identify the lineages. The western lineages (RE1, RE2 and RE3) were identified as *D. pseudotsugella* and the eastern Albertan material (RE4 and RE5) were identified as *D. reniculelloides*. We lacked larval host information for these specimens, the character often cited as a key diagnostic feature. Unfortunately even with larval host information, museum material and literature show that these species occur sympatrically and share larval host plants. Since diagnostic morphological characters were not found that would reliably separate these two species, they were combined in the diagnostic key. The of lack diagnostic morphological characters and unreliability of larval host and range for identification indicates that an extensive population study will be needed in areas of known overlap to elucidate this species complex and to assess the presence of distinct species boundaries.

Combining morphological characters with molecular data for pest identification has proved very effective (need lit). Morphological characters can be used to corroborate or refute molecular identification. Contamination of molecular samples is always a concern and having a suit of morphological characters to confirm identifications will reduce false identifications. Morphology can also be examined in the field, and does not rely upon lab facilities to obtain identification characters, speeding up identifications and the ability for forest managers to manage out breaks, as such morphological characters are an important component of diagnostic keys and compliments molecular data.

Pheromone Collections and Reared Material

Based on morphological identifications, four species, corresponding to four genetic lineages were collected at the GRC (*D. abietivorella, D. pentictonella, D.*

aurenticella, and *D. fordi*). Pheromone lures sampled three of the four species at this locality (all but *D. fordi).*

D. aurenticella was sampled once in the orchard using lure VI ((Z)-9-tetradecenyl acetate (100 ug)) (Table 3 & 4). Previous work also sampled *D. aurenticella* populations using this lure (Pasek and Dix 1989). Pheromone trapping at GRC caught a single specimen, suggesting that population numbers are low. The forewing of *D. aurenticella* lacks raised scales and is predominantly orange, corresponding to Type F (Table 3 & 4). No reared material was obtained in Chico, though previous collecting and literature document *Pinus ponderosa* and *P. attenuata* as larval host plants.

Pheromone trapping for *D. reniculelloides* or *D. pseudotsugella* was not conducted at Chico, CA. Previous work found pheromone lures composed primarily (Z)-9-tetradecenyl acetate (3 ug) attractive for *D. reniculelloides* (Grant *et al.* 1987). This compound is similar to lure VI, except lure VI had a concentration of 100 ug opposed to 3 ug in the *D. reniculelloides* lure. Previous work and museum material record *D. reniculelloides* in cones or needles from a variety of spruce (*Picea* sp.), Douglas-fir (*P. menziesii*), hemlock (*Tsuga* sp.) and firs (*Abies* sp.). *D. pseudotsugella* has been recorded primarily in cones and needles on Douglas-fir (*P. menziesii*), though also recorded on spruce (*Picea* sp.), hemlock (*Tsuga* sp.) and firs (*Abies* sp.).

D. pentictonella was attracted to a range of pheromone lures (Table 3 & 4). Lures VI and VII ((Z)-9-tetradecenyl acetate + (Z)-7-dodecenyl acetate (100 + 5 ug and 10 + 0.05 ug respectively) elicited the strongest response and large numbers of individuals were trapped. Variation in numbers of individuals throughout the season clearly indicates three peak emergence times: early May, June and October. These pheromone

lures attracted relatively high numbers of individuals and the specimens showed a broad range of wing phenotypes (Table 3 & 4). There was no correspondence between phenotypes and lures, except for the seasonal variation described previously. Literature and museum specimens describe the larvae as bud feeders, specializing on *Pinus ponderosa* and *P. contorta*. A single *D. pentictonella* specimen (RS1a) was reared from *Pinus brutia,* but this was an isolated case, and no other material was obtained from cones.

D. abietivorella was attracted to a range of lures (Table 3) though only VIII ((Z,E)-9,11-tetrdecadienyl acetate + (Z,E)-9,12-tetrdecadienyl acetate (100 + 1 ug)) attracted more than two individuals (Table 3 & 4). Relatively low numbers were caught using lure VII, particularly in comparison to the numbers captured with lures VI and VII (Fig. 12). *D. abietivorella* had a flight period from early May to October. The flight period and peak emergences for *D. abietivorella* attracted to lure VII were distinctly different than that of *D. pentictonella* attracted to lures VI and VIII (Fig. 12). This indicates that *D. abietivorella* lacks a distinct peak emergence time, instead multiple generations emerge throughout the year causing a broad flight period as shown in Fig. 12. Wing phenotypes show less variation than seen in *D. pentictonella*. The most common phenotype (Type I) lacked raised scales and red scales and was collected primarily with lure VII. Genitalia dissection confirmed the identification of these specimens as *D. abietivorella*. Large numbers of *D. abietivorella* specimens were also reared from *Pseudotsuga menziesii, Pinus ponderosa, P. brutia,* and *P. lambertiana* cones at GRC. Based this material, *D. abietivorella* was the primary agent causing damage to the cones in the Chico seed orchard, contradicting the numbers obtained with the pheromone lures.

It is likely a minor component of lure VII is absent, diminishing its attractiveness, thus reducing its catch numbers. Previous work on the sister species of *D. abietivorella* (*D. abietella* [Denis and Schiffermüller]) has demonstrated pheromone lure incompatibility, often due to a missing minor chemical component (need lit). Additional work will be needed to improve that attractiveness of the *D. abietivorella* lure so that pheromone catches accurately reflect population levels in the environment.

Species Group Relationships

In addition to improving the identification of *Dioryctria* in the Pacific Northwest, this study developed a preliminary phylogeny of species and species group relationships within the genus. Morphological identifications assigned the eight lineages to 6 previously described species groups (Table 3). These species groups were originally described based on genitalic variation and forewing differences (Mutuura and Munroe 1972). Species groups were erected to help clarify the variation seen between *Dioryctria* species and to simplify the taxonomy within the genus. The six species groups obtained in the survey and their related lineages were as follows: *zimmermani* Gr. (Lineages 1 & 2), *ponderosae* Gr. (Lineage 3), *schuetzeella* Gr. (Lineages 4 & 5), *aurenticella* Gr. (Lineage 6), *abietella* Gr. (Lineage 7), and *baumhoferi* Gr. (Lineage 8).

The full 2.3 kb of COI-COII resolved the monophyly of *Dioryctria* and improved the resolution of relationships between the species groups (Fig 13C), unlike the 475 bp fragment (Fig. 13B). The monophyly of the genus was well supported by bootstrap and Bremer support values (Fig. 14: Node A: 94%, 14), as well as by morphological characters described previously. The *abietella* Gr. was found to be sister to the rest of the species groups in the 2.3 kb phylogram, but this relationship was not supported by

bootstrap or Bremer support values (Fig. 14: Node D: 34%, 1). The *schuetzeella* Gr. and *aurenticella* Gr. showed a sister group relationship in the 2.3 kb tree, though this was also poorly supported (Fig. 14: Node B: 48%, 1). The *schuetzeella* Gr. showed increased support in the 2.3 kb phylogram (Fig. 14: Node C: 100%, 38). The *baumhoferi* Gr. showed a sister group relationship to the *zimmermani* Gr. + *ponderosae* Gr. and was moderately supported (Fig. 14: Node E: 77%, 4). The sister group relationship between *zimmermani* Gr. and *ponderosae* Gr. was well supported (Fig. 14: Node F: 100%, 17). The *zimmermani* Gr. was well supported (Fig. 14: Lineages 1 & 2: 100%, 17), similar to the 475 bp fragment (Fig. 13B: Lineages 1 & 2: 100%, 3). The two specimens of *D. okanaganella* formed the *ponderosae* Gr. (Lineage 8: 100%, 42) similar to the 475 bp data (Fig. 13B: 100%, 5). Increasing the number of characters in the data set has improved the resolution of the deeper phylogenetic relationships between the species groups, supporting test of data set size (Mitchell *et al*. 2000; Wahlberg 2003).

Several interesting trends were elucidated when the phylogenetic relationships of the six *Dioryctria* species groups were examined in light of morphological and ecological characteristics. Clade F (Fig. 14) was a well-supported sister group relationship between *zimmermani* Gr. and *ponderosae* Gr. This node was also supported by several synapomorphic morphological and ecological traits. Larvae in these groups all feed in the cambium of *Pinus* sp. and form pitch masses. Males have a constricted uncus (Figs. 7A, 8A, 9A) and a valve with a hooked apical projection (Figs. 7B, 8B, 9B).

Clade E (Fig, 14) was formed of three species groups ((*zimmermani* Gr. + *ponderosae* Gr.) + baumhoferi Gr.). This clade was moderately supported by the molecular data, but several additional synapomorphies also characterizes this clade.

Larvae all specialize on *Pinus* sp., though the feeding location on the tree is variable. Raised scales are present in several regions of the forewing (Figs. 6, 7, 8, 9). The species groups in this clade are also the most speciose in North America, with 30 species out of 40 described species classified under these three groups. Species of this group appear to specialize on one or two *Pinus* species. An adaptive radiation on to these various pine hosts might explain the disproportionate species diversity in these groups (need lit).

Based on the 2.3 kb phylogram, the *schuetzeella* Gr. and *aurenticella* Gr. formed a sister group relationship (Fig. 14). This relationship was not supported by the molecular data (Fig. 13C), but several morphological and ecological synapomorphies support this grouping. Both species groups lack raised scales, larvae feed primarily in cones, males lack both a constricted uncus (Figs. 3A, 4A) and an accessory spine (Fig. 3B, 4B).

Conclusions

Ultimately, combinations of mitochondrial, morphological and behavioral characters were needed to improve the identification of the *Dioryctria* species in the Pacific Northwest. This information demonstrated that *D. pentictonella,* was not responsible for cone damage and was collected exclusively by pheromone lures VI and VIII. Likewise, *D. abietivorella* was identified as the primary seed pest at the Genetic Resource Center in Chico, CA. This revelation, as well as the diagnostic characters that were obtained provides significant assistance for pest management in seed orchards.

Examination of multiple species in the Pacific Northwest has clarified both the identification of *Dioryctria* in the region, as well as provided a preliminary assessment of

the relationships between the species groups. Detailed examinations of all characters has improved the identification of *Dioryctria* species in the region, as well as improved management of these forest pests. The presence of two distinct species and the significant differences in their life history have profound consequences for future control efforts in this and other seed orchards. Accurate identification now allows efforts to be focused on development of lures that accurately reflects population numbers of *D. abietivorella*. Accurate estimations of population levels allows seed orchard managers to evaluate control methods, predict damage to seed crops and perhaps use mating-disruption to control this species.

The combined use of mtDNA and morphology in this species survey has been instrumental in delineating species, identifying diagnostic characters and for building a preliminary framework for a higher-level phylogeny of the species groups. The delineation and identification of these species will improve control of this pest through monitoring and possibly pheromone mating disruption. It was also imperative that the mtDNA lineages were examined for morphological variation in addition to genetic variation; otherwise identification and subsequent application to control would be impossible. The preliminary framework will allow future comparisons of additional species within these species groups to assess various morphological and ecological character evolution. Future work with this genus will need to focus on elucidating various species complexes, like between *D. reniculelloides* and *D. pseudotsugella* and increasing the sampling of species to build on the phylogenetic framework to further clarify the systematics of this genus.

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Table 3: Locality and collection information for *Dioryctria* specimens used in mtDNA surveys

Locality Data	Collection[1,4]	Flight Time	No.	Collector, Year	Haplotype
abietella Group					
D. abietivorella					
CA: Butte Co., Chico	Pheromone – VII, IV, III, V, VI	Late May - Sept	14	C. Rudolf, G. Grant (1998-2001)	AB1[3]
CA: Butte Co., Chico	Cone: Pp, Pb, Pl, Df		42	C. Rudolf (1995, 2000-2001)	AB1
CA: Butte Co., Chico	MV-light	June	6	A. Roe (2001)	AB1
CA: Placer Co., Foresthill	Cone: Pp		3		AB1
OR: Lane Co., Cottage Grove, Dorena Tree Center	Cone: Pm, Lo		3	J. Berdeen (2001)	AB1
OR: Clackamas Co., Colton, Horning Tree Center	Cone: Df		1	B. Willhite (2001)	AB1
CA: Butte Co., Chico	Cone: Pb, Pl, Df		6	C. Rudolf (2000-2001)	AB2
CA: Butte Co., Chico	MV-light	June	1	A. Roe (2001)	AB2
CA: Butte Co., Chico	Cone: Df		1	C. Rudolf (2001)	AB3
aurenticella Group					
D. aurenticella					
CA: El Dorado Co., Placerville	MV-light	June	2	A. Roe (2001)	OS1[3]
CA: Butte Co., Chico	MV-light	June	2	A. Roe (2001)	OS2
CA: El Dorado Co., Placerville	MV-light	June	1	A. Roe (2001)	OS2
schuetzeella Group					
"*D. pseutotsugella*"					
OR: Benton Co., Corvalis	UV-light	July	2	J. Adams (2001)	RE1[3]
OR: Benton Co., Corvalis	UV-light	July	1	J. Adams (2001)	RE2
OR: Benton Co., Corvalis	UV-light	July	2	J. Adams (2001)	RE3
"*D. reniculelloides*"					
CAN: AB: Fort MacMurray	UV-light	July	1	A. Roe (2001)	RE4[3]
CAN: AB: Fort MacMurray	UV-light	July	1	A. Roe (2001)	RE5
baumhoferi Group					
D. pentictonella					
CA: Butte Co., Chico	Pheromone – VI, VIII, II, I, III, IV, IX	May – October	52	C. Rudolf, G. Grant (1998-2001)	RS1a[3]
CA: Siskiyou Co., Ball Mt.		September	2		RS1a
CA: Butte Co., Chico	Cone: Pb	September	1	C. Rudolf (1997)	RS1a
CA: El Dorado Co. Placerville	MV-light	August	1	A. Roe (2001)	RS1a
CA: Butte Co., Chico	Pheromone – VI, II, VIII, III	May - June	16	C. Rudolf, G. Grant (1998-2001)	RS1b[3]
CA: Alameda Co. Berkeley	UV-light	June	1	FAH Sperling (1998)	RS1b
CA: Butte Co., Chico	Pheromone - IV	May	1	C. Rudolf, G. Grant (1998)	RS1c
CA: Butte Co., Chico	Pheromone - VIII	September	1	C. Rudolf, G. Grant (2000)	RS1e
CA: Siskiyou Co., Ball Mt.	UV-light	September	1	Light	RS1f
CA: El Dorado Co., Placerville	MV-light	June	1	A. Roe (2001)	RS1d[3]
zimmermani Group					
D. cambiicola					
OR: Medford	Bark: Df		1	J. Berdeen (2001)	RS2a
OR: Medford	Bark: Df		1	J. Berdeen (2001)	RS2b[3]
D. fordi					
CA: Butte Co., Chico	MV-light	June	3	A. Roe (2001)	RS2c[3]
CA: Butte Co., Chico	MV-light	October	2	A. Roe (2002)	RS2h
CA: Butte Co., Chico	MV-light	October	2	A. Roe (2002)	RS2i
CA: Butte Co., Chico	MV-light	October	1	A. Roe (2002)	RS2g
ponderosae Group					
D. okanaganella					
CA: El Dorado Co., Placerville	MV-light	June	1	A. Roe (2001)	RS2d
CA: El Dorado Co., Placerville	MV-light	June	1	A. Roe (2001)	RS2e[3]
CA: El Dorado Co., Placerville	MV-light	June	1	A. Roe (2001)	RS2f[3]

[1]Host records from material reared to adult or extracted as larvae
[2]Host abbreviations: Pb: *Pinus brutia*; Pl: *Pinus lambertiana*; Pp: *Pinus ponderosa*; Pm: *Pinus monticola*; Df: *Pseudotsuga menzesii*; Lo: *Larix occidentalis*
[3]Haplotypes sequenced for 2.3 kb of COI-COII

Table 4: *Dioryctria* mtDNA haplotypes, wing phenotypes and associated pheromone lures collected at the Genetic Resource Center at Chico, California

Haplotype	Species	No.	Lure	Components[1](Dose [ug])	Wing Phenotypes[3]
AB1[2]	*D. abietivorella*	9	VII	Z (100) + E (1)	I*, D
		2	IV	Z (100)	E,H
		1	III	T (10) + D (1)	-
		1	V	T (100)	-
		1	VI	T (100) + D(5)	I
OS1[2]	*D. aurenticella*	1	VI	T (100)	F
RS1a[2]	*D. pentictonella*	42	VI	T (100) + D (5)	A*, B, C, E, H, G
		2	II	T (100)	A, C
		2	III	T (10) + D (1)	D, G
		3	VIII	T (10) + D (0.05)	C, H, G
		1	I	T (10)	A
		1	IX	T (1) + D (0.05)	-
		1	IV	Z (100)	H
RS1b[2]	*D. pentictonella*	5	VI	T (100) + D (5)	E*, H
		2	VIII	T (10) + D (0.05)	D, G
		1	II	T (100)	D
		1	III	T (10) + D (1)	A
RS1c[2]	*D. pentictonella*	1	VIII	T (10) + D (0.05)	-
		1	IV	Z (100)	G
RS1e	*D. pentictonella*	1	VIII	T (10) + D (0.05)	B

[1]Chemical names of *Dioryctria* pheromone lure components:
T: (Z)-9-tetradecenyl acetate
D: (Z)-7-dodecenyl acetate
Z: (Z,E)-9,11-tetradecadienyl acetate
E: (Z,E)-9,12-tetradecadienyl acetate
[2]Wing scored based on previously described phenotypes (J. Stein, unpublished)
[3]Haplotypes sequenced over 2.3 kbp COI-COII
*Most common wing phenotypes
- Wings too damaged to identify phenotype

Table 5: Mitochondrial DNA primers used in surveying *Dioryctria* species over 2.3 kb of COI-COII in the Pacific Northwest

Primer Name	Direction and Location (3' end)[1]	Sequence (5' – 3')
K698	TY-J-1460	TAC AAT TTA TCG CCT AAA CTT CAG CC
RonV	C1-J-1751	GGA GCT CCA GAT ATA GCT TTC CC
K699	C1-N-1840	AGG AGG ATA AAC AGT TCA (C/T)CC
K808	C1-N-1840	TGG AGG GTA TAC TGT TCA ACC
Jerry*	C1-J-2183	CAA CAT TTA TTT TGA TTT TTT GG
JerryV*	C1-J-2183	CAA CAT TTA TTT TGA TTC TTT GG
Nancy	C1-N-2191	CCC GGT AAA ATT AAA ATA TAA ACT
K525	C1-N-2329	ACT GTA AAT ATA TGA TGA GCT CA
Brian	C1-J-2495	CTT CTA TAC TTT GAA GAT TAG G
MilaIII*	C1-N-2659	ACT AAT CCT GTG AAT AAA GG
George	C1-J-2792	ATA CCT CGA CGT TAT TCA GA
GeorgeIII	C1-J-2792	ATA CCT CGG CGA TAC TCT GA
GeorgeV	C1-J-2792	ATA CCT CGA CGA TAT TCC GA
PatII	TL2-N-3014	TCC ATT ACA TAT AAT CTG CCA TAT TAG
Pierre	C2-J-3183	AGA GCC TCT CCT TTA ATA GAA CA
Marilyn	C2-N-3389	TCA TAA GTT CA(A/G) TAT CAT TG
MarilynII	C2-N-3389	TCA TA(T/A) CTT CA(A/G) TAT CAT TG
MarilynIII	C2-N-3389	TCA TAT CTT CAG TAT CAC TG
Preston	C2-J-3570	GCA ACA GAT GTT ATT CAC TCT TG
Eva	C2-N-3782	GAG ACC ATT ACT TGC TTT CAG TCA TCT

[1]Following Simon *et al*. 1994: J/N: Majority/Minority (equivalent to sense/antisense for COI-COII). Posistion relative to *Drosophila yakuba* (Clary and Wolstenholme 1985).
*Primer combination used for the 475 bp fragment.

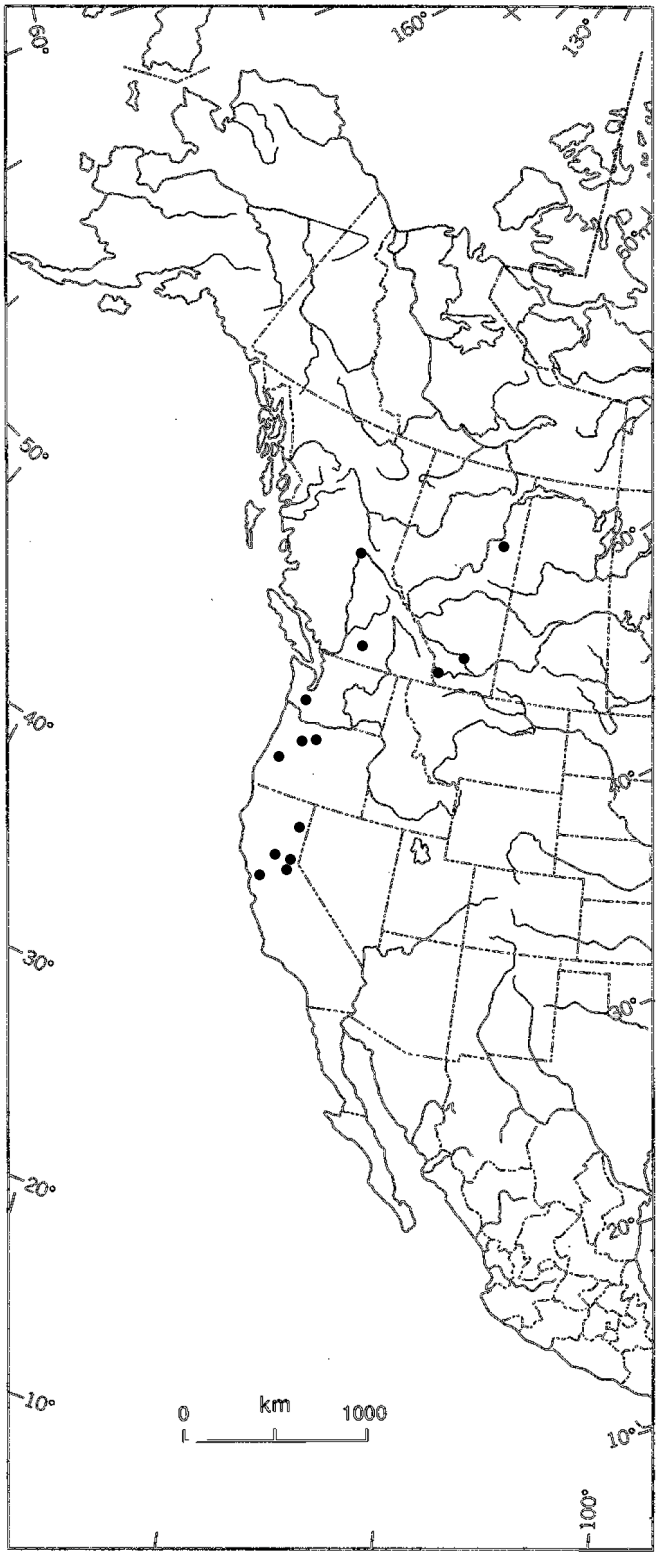

Fig. 11: Map of specimen localities examined for treatment of Pacific Northwest *Dioryctria* species

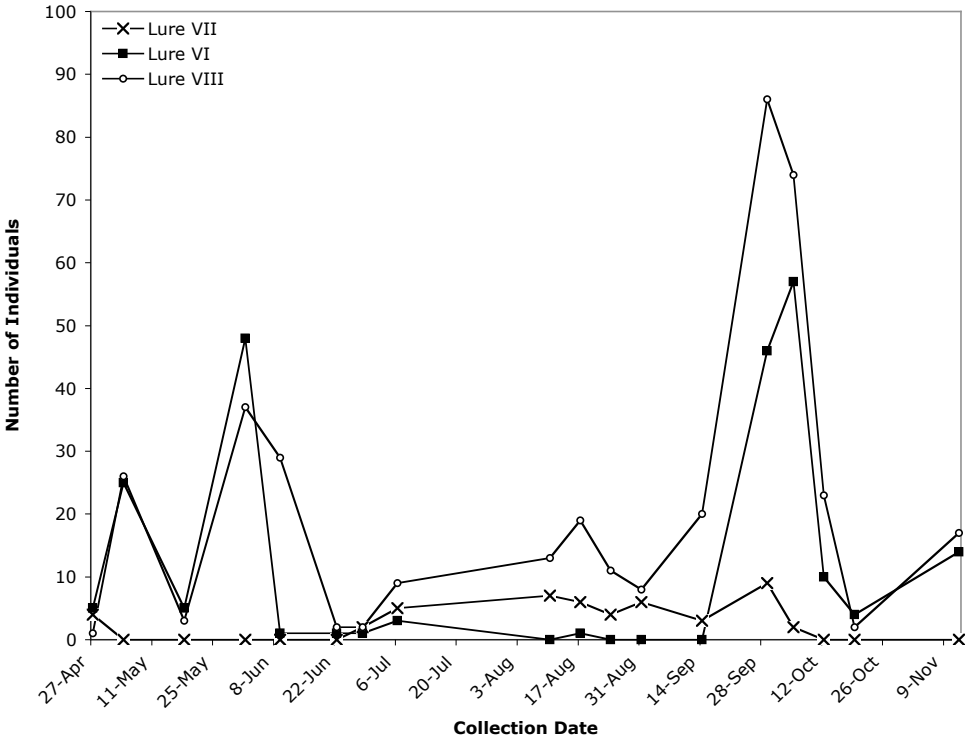

Fig. 12: Pheromone captures throughout 2000 season for lures VI, VII and VIII.

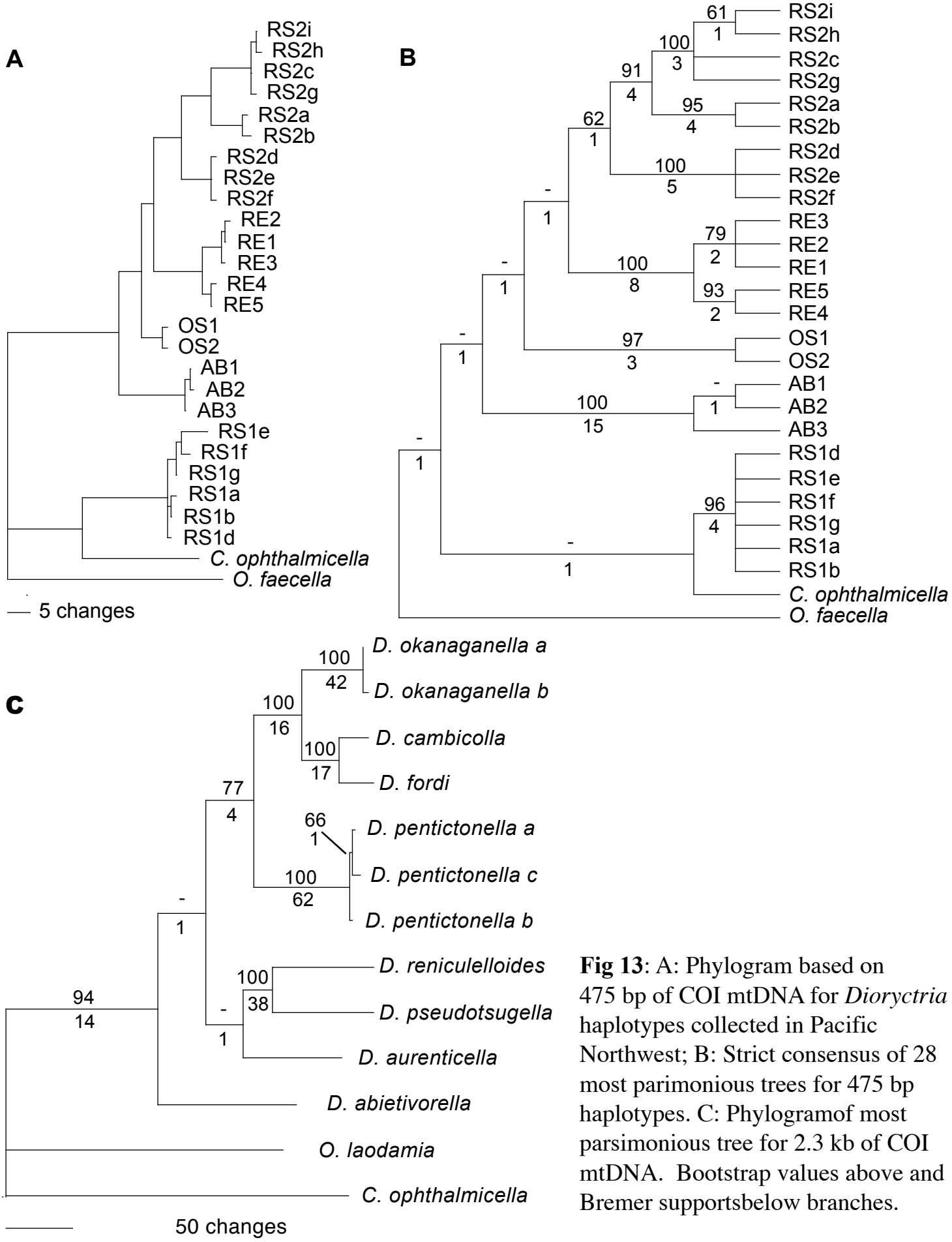

Fig 13: A: Phylogram based on 475 bp of COI mtDNA for *Dioryctria* haplotypes collected in Pacific Northwest; B: Strict consensus of 28 most parimonious trees for 475 bp haplotypes. C: Phylogramof most parsimonious tree for 2.3 kb of COI mtDNA. Bootstrap values above and Bremer supportsbelow branches.

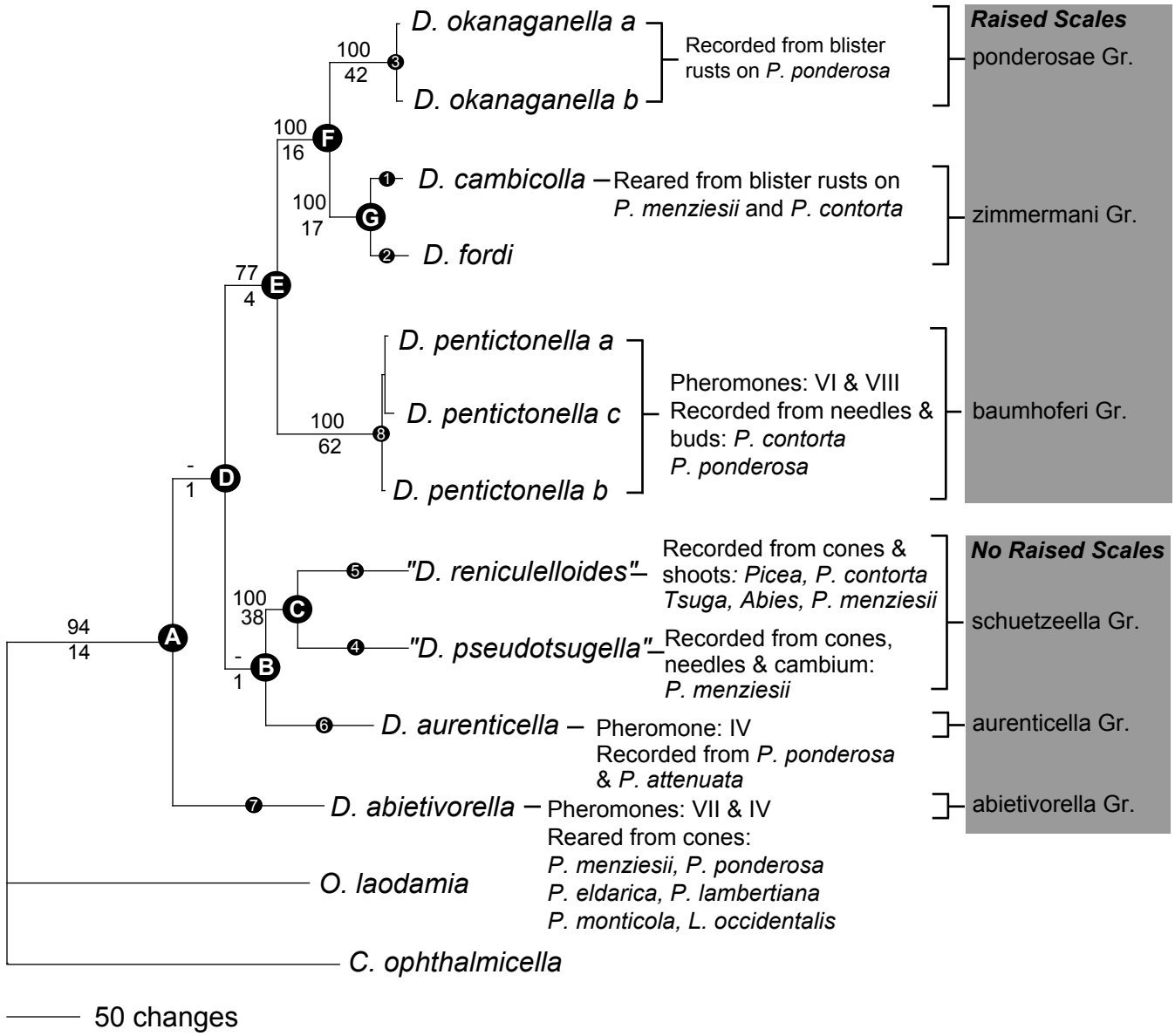

Fig 14: Single most parsimonious phylogram of 2.3 kb dataset. Lineages are labled 1-8. Nodes are labled A-G. Key features of each lineage are indicated on the tree. Species groups are shsded based on presence or absence of raised scales.