Chemical Ecology and Mechanisms of Reproductive Isolation in Ambrosia Beetles

by

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ABSTRACT

Species-specificity in signals that mediate reproductive interactions are of adaptive advantage in avoiding the cost of mismating and potential low fitness through hybridization. Phytophagous insects retain species-specificity in part by multicomponent sex pheromones synergized or otherwise enhanced by host chemicals. For four sympatric species of *Trypodendron* spp. (Coleoptera: Scolytidae) in British Columbia, coupled gas chromatographic-electroantennographic detection analysis of volatiles from beetles, hosts and non-hosts revealed several antennally active compounds. These were tested in field bioassays for their behavioural significance as reproductive isolating mechanisms in finding hosts, attracting conspecifics and repelling heterospecifics. The *ESR* and *ZRR* stereoisomers of tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol (tirathol) were identified as the aggregation pheromone of *T. betulae*. Responses were synergistically increased when the pheromone was combined with the birch host volatile conophthorin and ethanol. Use of tirathol isolates *T. betulae* semiochemically from three other sympatric *Trypodendron* species, which were shown to use the pheromone (+)-lineatin. *Trypodendron retusum* responded to a synergistic combination of lineatin, ethanol, and the aspen host volatile salicylaldehyde, with apparent phylogeographic variation between coastal and interior populations. Salicylaldehyde repelled coniferophagus *T. lineatum* and *T. rufitarsus*, while *T. retusum* was repelled by the conifer volatile *alpha*-pinene. Examination of the symbiotic fungal associations of the four native ambrosia beetles and the established exotic *T. domesticum* revealed overlapping combinations of fungal isolates among the genera *Ophiostoma*, *Ambrosiella*, and *Ceratocystiopsis*, with no evidence of co-evolution between fungi and their beetle vectors. Isolation techniques,

anatomic locations, and insect life stage were crucial in determining fungal partners. The construction of a molecular phylogeny based on COI and a *Wingless* regions of the North American and European *Trypodendron* species and two other species in the tribe Xyloterini placed the European angiosperm-infesting *T. signatum* ancestral to all other *Trypodendron* spp. North American angiosperm specialists, *T. retusum* and *T. betulae* are derived from coniferous generalists. The phylogeny does not allow for conclusions to be drawn about the evolution of pheromones or fungal associates as reproductive isolating mechanisms.

DEDICATION

To my late father who passed away when I was carrying twins and preparing this thesis. I wish he could be here to see me as a mother and as a Ph.D.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 General Introduction

Unraveling the mechanisms of reproductive isolation contributes to answering one of the most enduring scientific challenges, to understand speciation. Differences in mating signals can mediate reproductive isolation and promote speciation among populations (Andersson, 1994; Boughman, 2001; Higashi et al., 1999; Maynhard Smith, 1966; Panhuis et al., 2001). The extraordinary diversity of auditory, visual, and olfactory communication signals used in the animal kingdom may be the result of selection for signals that are different from others produced in the same environment (Loftus-Hill and Littlejohn, 1992; Rodríguez et al., 2004; Roelofs, 1995; Roelofs and Cardé, 1974; Ryker and Rudinsky, 1976). Bark and ambrosia beetles (Coleoptera: Scolytidae and Platypodidae) utilize elaborate semiochemical communication signals that transmit messages announcing the location and suitability of hosts and availability of mates (Birch, 1974, 1984; Borden, 1977, 1982a; 1985; Byers, 1989; Vité and Francke, 1976; Wood, 1982).

Semiochemicals are natural compounds produced and released by individuals of a species, which elicit a behavioral response in members of the same or different species (Nordlund, 1981). In insects the ecological role of semiochemicals in mediating interactions with con- and heterospecifics and the environment is well investigated, but more so is the significance of semiochemicals in manipulating insect behaviour because of the enormous economic impact of many pest insects. Semiochemicals which are used in intraspecific communication are referred to as pheromones. Behavioral responses to pheromones include searching for mates by one sex (sex pheromones), aggregation of both sexes at a host plant (aggregation pheromones), and dispersal of one or both sexes

away from a specific area (marker or antiaggregation pheromones, respectively). Semiochemicals used in interspecific communication are referred to as kairomones when the species receiving the chemical message benefits, allomones when the emitter of the chemical message benefits at the expense of the receiver, and synomones when there is mutual benefit.

After the discovery of the first sex pheromone, bombykol, from the silkworm, *Bombyx mori* (L.), it was generally thought that each insect species produces and responds to single compound (Karlson and Butenandt, 1959). It is now known that multi-component blends of pheromones or combinations of pheromones and plant compounds are required to maintain reproductive isolation of closely related species, while single component systems are the exception rather than the rule (Cardé et al., 1979). There is considerable overlap with regard to the functions of a compound or a blend, as the same compound may serve both intra- and interspecific functions. For example, frontalin serves as an aggregation pheromone to the southern pine beetle, *Dendroctonus frontalis* Zimmerman and as a kairomone to its predator, *Thanasimus dubius* (F.) (Vité and Williamson, 1970). Members of one species may release a component which is attractive to conspecifics, but inhibitory to related species (Evenden et al., 1999; Seybold et al., 2000). The focus of research on attractive host-produced kairomones for bark beetle (Byers 1995) and on beetle-produced, aggregation pheromones (Borden 1985) has more recently been expanded to embrace the role of negative information provided by non-hosts during host selection (Borden et al., 1998; Huber et al., 2000; Schroeder, 1992; Tømmerås and Mustaparta, 1989; Zhang and Schlyter, 2004). Insect plant interactions may also involve compounds produced by microbial mutualists, such as symbiotic fungi

(Paine et al., 1997), which also can aid an insect by killing its host (Raffa et al., 1993) or providing food (Landolt and Phillips, 1997).

Ambrosia beetles are named after their habit of feeding their young with symbiotic fungi, the 'ambrosia'. The name was first applied to the glistening lining of the tunnels before its fungal nature was known (Schmidberger, 1836). While studying the ambrosia beetle *Xyleborus* [=*Anisandrus*] *dispar* (F.) Hartig in 1844 recognized the fungus and named it *Monilia candida* Hansen (Baker, 1963).

The ambrosia beetle genus *Trypodendron* Stephens consists of 14 species worldwide of which four (*T. betulae* Swaine, *T. retusum* (LeConte), *T. rufitarsus* (Kirby), *T. scabricollis* LeConte) are North American, one (*T. lineatum* (Olivier)) is holarctic, three species (*T. signatum* (F.), *T. domesticum* (L.), *T. laeve* Eggers) are Eurasian in distribution (Wood and Bright, 1992). All species are very similar in their biology. They are xylomycetophagous, monogamous and normally univoltine. In B.C., *T. lineatum* is polyphagous, colonizing numerous conifers species in it range (Wood and Bright, 1992), while *T. rufitarsus* appears to prefer *Pinus* spp. (pers. obs.) but also infests other conifers in its range (Wood and Bright, 1992). *Trypodendron retusum* attacks two *Populus* species trembling aspen, *Populus tremuloides* Michx, and black cottonwood *Populus trichocarpa* Torr. & Gray ex Hook, but according to my personal observations and Bright (1976) preferentially infests trembling aspen. *Trypodendron betulae* is reported in paper birch, *Betula papyrifera* Marsh. and *Betula lenta* L., and in B.C. attacks only the former species.

The conventional view of ambrosia beetles' typical hosts is that of unhealthy, physiologically stressed, dying or dead trees, in forestry sometimes called unthrifty trees, or injured parts of living trees (Wood, 1982b). However, some species are specialists on

living trees, and recent observations report some *Trypodendron* spp. in Europe and B.C. attacking standing apparently healthy trees (Appendix 1). Adults begin to fly in the spring when temperatures reach a critical level, and disperse to find suitable hosts. For example *T. rufitarsus* flies when the maximum temperature is as low as 8°C, and the ground is still covered in snow (pers. obs.), while *T. lineatum* does not start flying until the ambient temperature reaches 15.5°C (Chapman and Kinghorn, 1958; Rudinsky and Daterman, 1964).

Females initiate the galleries and bore directly into the sapwood. Mates are attracted during the dispersal and gallery initiation phase and mating usually occurs outside of the entrance hole; egg laying begins after niches are excavated along galleries. With each egg, females also inoculate the galleries with spores of symbiotic ambrosia fungi, which are transported in specialized prothoracic chambers called mycangia (Francke-Grosmann, 1958). After germination, the fungus penetrates and grows on the xylem tissue. The brood and the fungi are tended bi-parentally, an adaptation apparently necessary for successful breeding (French and Roeper 1972, Kirkendall et al., 1997). Ambrosia beetle larvae of all ages feed exclusively on ambrosia fungi (Batra, 1966; Beaver, 1989; Francke-Grosmann, 1967). Brood adults emerge in the late summer to find overwintering sites in forest litter and duff and in the basal area of large trees and stumps (Borden, 1988; Bright, 1976; Wood, 1982b). However, *T. betulae* has also been observed overwintering in host trees (L. Humble, Pacific Forestry Centre, Natural Resources Canada, Victoria, B. C., pers. comm.).

Ambrosia beetles play an important role, both ecologically as one of nature's recyclers (Borden, 1984) and economically (McLean, 1985), because the beetles' dark

stained galleries in the sapwood degrade logs and wood products. The estimated annual economic impact of ambrosia beetles, principally assumed to be *T. lineatum*, ranges from $95 to $189 million (Can.) on B.C. coast alone (Lindgren and Fraser, 1994a). Because of ambrosia beetles' cryptic lifestyle, traditional pesticide sprays are ineffective and environmentally undesirable. Management of ambrosia beetles currently consists mainly of population suppression through semiochemical-based mass trapping beetles in the spring and summer coupled with management of timber inventories to minimize exposure to attack (Borden et al., 2001). Exploitation of semiochemicals as management tools requires a thorough understanding of the target species and associated organisms. A better understanding of host selection and attack by all species may lead to more effective integrated use of aggregation pheromones, attractive host kairomones and putative non-host repellents in managing insects. Until the start of my research the focus species in ambrosia beetle management in B.C. was *T. lineatum*, while the other three species were overlooked. Except for recent studies by Hoover et al. (2000) and Lindgren et al. (2000), *T. rufitarsus* was probably missed because of its' early and short flight period, and the two angiosperm-attacking beetles because of the relatively minor role of hardwood products in the B.C. forest industry to date.

The overall scope of this thesis was to determine factors that mediate reproductive isolation of the four sympatric *Trypodendron* spp. in B.C. I tested the hypotheses that these species are in part reproductively isolated by pheromones in combination with host or non-host volatiles, and that fungal associations have co-speciated with the beetles. The construction of a molecular based phylogeny of the North American and European *Trypodendron* species provided a tool to test whether phenotypic semiochemical and

fungal specificity parallels the beetles' phylogenetic relationships.

2 Chemical Ecology of *Trypodendron* species

2.1 Introduction

The sexual behaviour of phytophagous insects is integrated with chemical communication strategies that optimize foraging, habitat selection and reproduction (Landolt and Phillips, 1997). Highly species-specific chemical signals may mediate behaviour-based reproductive isolation among sibling species (Landolt and Phillips, 1997; Tumlinson et al., 1974), reducing the likelihood of mismating and possibly hybridization (Dobzhansky, 1940). Natural selection will favour insects that can perceive specific chemicals used to locate acceptable hosts and mates (Byers, 1995). To an insect, the environment is filled with a plethora of potentially detectable sensory cues. However, an insect possesses only a limited quantity of olfactory receptors and can consequently detect only a limited suite of volatile compounds (Moeck, 1968).

Host chemicals may directly mediate host selection by scolytid beetles or may synergize the responses to aggregation pheromones (Borden, 1977, 1984, 1993; Wood, 1982a). Chemical cues that beetles encounter during host and mate selection are complex (Borden et al., 1986), and include attractive volatiles from hosts, volatiles from hosts accentuated by pheromones of conspecifics, repellent volatiles from non-hosts, and volatiles from non-hosts accentuated by pheromones emitted by sympatric heterospecific beetles (Ayres et al., 2001; Poland and Borden, 1998; Rankin and Borden, 1991). If sympatric and syntopic insect species have identical pheromones, e.g. *Lambdina athasaria* (Walker) and *L. pellucidaria* (Grote and Robinson) (Duff et al., 2001), other isolating mechanisms are needed to ensure reproductive isolation.

For those scolytid beetles species with pheromones, there are two distinct phases

of host selection: primary, which involves only a few beetles selecting a susceptible host tree, and secondary, which involves the majority of beetles as they respond to the pheromones produced by successful primary beetles or a blend of pheromones and synergistic host volatiles (Borden, 1982b, 1985). The dispersal, host selection and mate finding phases comprise the most important period in a scolytid beetle's life in terms of potential fitness consequences (Atkins, 1966a; Gries et al., 1989). Because *Trypodendron* species require high-quality hosts that are an ephemeral and evanescent resource, random landing on host and non-host trees followed by close-range testing for suitability (Elkinton and Wood, 1980; Moeck et al., 1981) is a less plausible strategy than a strong primary attraction response (Gries et al., 1989). Closely related beetles in the family Scolytidae display abundant evidence for pheromone specificity (Lanier and Wood, 1975) and for synomones that deter sympatric heterospecifics and attract conspecifics (Borden, 1997). All *Trypodendron* spp. in British Columbia are sympatric, contingent on host tree distributions in local areas. Pheromone-based reproductive isolation for the genus is not documented and other behavioural barriers that prevent interspecific mating are not known.

The occurrence of a female-produced aggregation pheromone in *T. lineatum* was discovered by Rudinsky and Daterman (1964). The pheromone was isolated from female-produced frass, identified as 3,3,7-trimethyl-2,9-dioxatricyclononane, and given the trivial name lineatin (MacConnell et al., 1977). The absolute configuration of the active enantiomer was determined from synthetic enantiomers to be 1*R*,4*S*,5*R*,7*R*-(+)-lineatin (Slessor et al., 1980). (-)-Lineatin has no inhibitory effect and the racemic mixture is as attractive as the (+) enantiomer (Borden et al., 1980b). It is produced in the hindgut-

malpighian tubule region (Borden and Slater, 1969) and is highly attractive to both sexes in the field (Borden et al., 1979; Klimetzek and Francke, 1980; Vité and Bakke, 1979). *Trypodendron lineatum* exhibits a strong primary attraction response (Chapman, 1962). It is attracted to ethanol alone (Cade et al., 1970; Moeck, 1970) as an indicator of stressed or dying trees (Graham, 1968; Kelsey and Joseph, 1999, Kelsey and Joseph 2003) and to blends of *alpha*-pinene and ethanol (Borden et al., 1980b; Nijholt and Schönherr, 1976; Vité and Bakke, 1979). Ethanol and *alpha*-pinene act synergistically with lineatin in European populations (Klimetzek and Francke, 1980; Vité and Bakke, 1979), but α-pinene is not required for maximum response in England (Borden et al., 1982), and may be inactive in North America (Salom and McLean, 1988). Ethanol is inhibitory at high concentrations in North America (Borden et al., 1982), and is reportedly either inert (Borden et al., 1982; Salom and McLean, 1988) or synergistic (Shore and McLean, 1983) at low concentrations.

Since (+)-lineatin was established to be an aggregation pheromone for *T. lineatum*, it has been found to occur in other *Trypodendron* spp. (Schurig et al., 1982) and to have a similar function (Klimetzek et al., 1981). In B.C., *T. rufitarsus* and *T. retusum,* but not *T. betulae,* were also found to respond to (+)-lineatin (Hoover et al., 2000; Lindgren et al., 2000), but production of (+)-lineatin was not verified.

2.2 Objectives

The general objective of this part of my dissertation was to investigate the semiochemical-based mechanisms of reproductive isolation among the four native sympatric ambrosia beetles species in the genus *Trypodendron* in British Columbia. The

specific objectives were to:

1) determine the pheromones of *T. rufitarsus*, *T. retusum*, and *T. betulae*;
2) identify antennally-active volatiles emanating from host and non-host tree species that could be used in host selection decisions by the beetles; and to
3) conduct field bioassay experiments with emphasis on *T. retusum* to test the behavioural activity to pheromones alone and in combination with host and non-host volatiles in order to elucidate species-specific olfactory responses that could contribute to semiochemical-based reproductive isolation.

2.3 Methods

2.3.1 Collection of Beetles and Host Material, and Capture of Volatiles

Ambrosia beetles were collected in traps, on hosts, or in cut bolts from attacked host trees in various locations in B.C. (Table 2.1). In the laboratory *T. betulae* and *T. retusum* were excised from host logs by cutting 5 cm thick discs with a chainsaw and then carefully opening the gallery by chisel and hammer. Beetles were sorted by sex using sexual dimorphism of the frons (Wood, 1982b) and held separately in plastic Petri-dishes with moist filter paper for a maximum of 4 h in a cool, shaded place or in a refrigerator at a temperature of approximately 5°C. Beetles were then placed in Pyrex® glass tubes (1.2 cm OD × 18 cm long) containing moist tissue paper. Charcoal-filtered air was drawn through the tubes containing the beetles and then through a glass column (6 mm OD × 15 cm long) containing 3 cm of Porapak Q (50-80 mesh, Waters Association Inc., Milford, MA 01757) (Byrne et al., 1975; Gries et al., 1992), with approximately 1.5

TABLE 2.1 Method, location, and dates of collection, and numbers of collected beetles used in aerations of *Trypodendron* species.

Species	Method	Location (B.C.)	Collection dates	No. of beetles
T. lineatum	lineatin baited trap	Brookmere	23-25 April, 1999	>400 females >400 males
T. rufitarsus	lineatin baited trap	Brookmere	23-25 April, 1999	>100 females >100 males
T. retusum	removed while walking on aspen trees or excised from aspen logs	Brookmere	21 April, 1999	80 females 86 males
T. betulae [a]	excised from birch log	Mission	28 April, 1998	92 females 100 males
T. betulae [b]	excised from birch log	Maple Ridge	06 April, 1999	56 females 55 males

[a] Aeration for initial collection of beetle-produced volatiles.

[b] Aeration to demonstrate production of identified pheromone.

L/min using a water aspirator. Beetles were aerated in this fashion at a temperature of 23°C for 3-7 days, at which point most beetles had died. Captured volatiles were recovered by elution of the Porapak Q trap with 150 mL of distilled pentane (Pierce et al., 1981).

Host and non-host trees (Table 2.2) were felled with a chain saw, cut into bolts, 60-100 cm long, and either used within 24 h of being transported to the laboratory or stored at a temperature of -12°C for no longer than 7 days. Bark and sapwood were removed with an axe, resulting in pieces 5-20 cm long and 1-5 cm thick. Approximately 3-4 kg of wood and bark were placed in airtight, 10 L plastic chambers maintained at 23°C. Charcoal-filtered air was drawn through the chamber for 3-7 days at 3-4.4 L/min using a water aspirator. Volatiles were captured and eluted as described above. For initial analyses, the volatiles from several host or non-host trees were combined. For instance, for testing against *T. lineatum* and *T. rufitarsus,* host volatiles from Douglas-fir, western hemlock, interior spruce, and lodgepole pine were combined and non-host volatiles from bigleaf maple, black cottonwood, red alder, and paper birch were combined.

2.3.2 Antennal Responses to Tree and Beetle Volatiles

The ability of beetles to detect host, non-host, and beetle-produced volatiles was analyzed using coupled gas chromatographic-electroantennographic detection (GC-EAD) analysis (Arn et al., 1975). These analyses were conducted by Ms. Regine Gries, Department of Biological Sciences, Simon Fraser University. In brief, volatiles were simultaneously analyzed by an electrophysiological antennal detector (EAD) with a glass microelectrode inserted into the antennal club of males and females of each beetle

TABLE 2.2 Species of tree, diameter at breast height (dbh = 1.3 m), sample mass, location and dates of felling and volatile collection for host and non-host material used in aerations.

Tree species (dbh in cm)	Location (B.C.)	Date of eration (date of cutting)	Mass (g)
Douglas-fir, *Pseudotsuga menziesii* (Mirb.) (21.3)	Mesachie Lake, Vancouver Island	22-27 January, 1998 (December, 1997)	3825
western hemlock, *Tsuga heterophylla* (Raf.) Sarg. (16.5)	Burnaby Mtn.	24-27 February, 1998 (December, 1997)	3104
interior spruce, *Picea engelmannii* Parry (21.1)	Whipsaw Road, near Princeton	10-14 April, 1998 (31October, 1997)	3542
lodgepole pine, (fresh) *Pinus contorta* Dougl (17.0)	Lytton	16-20 April, 1998 (16 April, 1998)	4125
lodgepole pine (aged) (21.6)	Whipsaw Road, near Princeton	10-14 April, 1998 (3 January, 1998)	4491
bigleaf maple, *Acer macrophyllum* Pursh (16.6)	Mission	4- 7April, 1998 (December 1997)	3489
black cottonwood, *Populus trichocarpa* Torr. & A. Gray (19.7)	Burnaby Mtn.	4-7 March, 1998 (19 February, 1998)	2625
red alder, *Alnus rubra* Bong. (24.4)	Burnaby Mtn.	4-7 April 1998 (February, 1998)	4141
paper birch, *Betulae papyrifera* Marsh. (25.3)	Mission	21-24 February, 1998 (January, 1998)	3630

species, and by a flame ionization detector (FID) (Gries, 1995) employing a Hewlett Packard (HP) 5890 gas chromatograph (GC) equipped with a fused silica column (30 m × 0.25 or 0.32 mm ID) coated with DB-23 or DB-210 (J&W Scientific, Folsom, CA 95630) under splitless conditions and a different temperature program in different runs using helium as the carrier gas. GC-EAD analyses were carried out on live adult *T. lineatum*, *T. rufitarsus*, *T. retusum*, *T. betulae* and *T. domesticum* from B.C. and *T. domesticum* (L.) and *T. signatum* (F.) from Belgium, provided by JC Grégoire, (Universite Libre de Bruxelles, Belgium). A custom built amplifier with a passive low-pass filter and cut-off frequencies below approximately 0.02 Hz and 10 kHz was used to amplify antennal responses. Compounds eliciting an antennal response were subjected to GC-mass spectrometry (GC-MS) (Varian Saturn Ion Trap). Spectral comparison with authentic standards and calculated retention indices (van den Dool and Kratz, 1963) were used to identify compounds. The enantiomeric ratios of chiral compounds were determined using a Cyclodex B column (30 m × 0.25 mm ID, J&W Scientific) and an isothermal temperature program of either 80°C or 120°C and split analyses. Peaks in aeration extracts were compared to standards obtained from commercial or natural sources.

2.3.3 Field Trapping Experiments

Field trapping experiments within established populations of *T. lineatum*, *T. rufitarsus, T. betulae* and *T. retusum* in B.C. were conducted to determine the bioactivity of various compounds. Coastal and interior populations of *T. lineatum* and *T. retusum* were tested because of past indications of geographically distinct behaviour. All experiments utilized 12-unit multiple-funnel traps (Lindgren, 1983) (Phero Tech Inc.,

Delta, B.C.) placed 15-20 m apart (to minimise the effects of adjacent treatments) along logging roads, trails, and cut-block margins, with treatment combinations in randomized complete linear blocks. This procedure has become a standard method for field testing beetle responses to semiochemicals (Lindgren et al., 1983). Each of 10 experiments (Table 2.3) consisted of a positive control utilizing commercially available pheromone or a pheromone and host volatile combination that I determined to be attractive, an untreated control, and several experimental treatments. In all experiments, semiochemicals were hung on funnel numbers 4-6 within the central space of the vertically-aligned funnels of the traps. A small block of dichlorvos-impregnated plastic (Vapona No-pest® strip, Monsanto Canada Ltd., Mississauga, Ontario) was placed in each collecting cup to minimise escape by and predation on captured beetles. Insects were collected and frozen until sexed (except for Experiment 5) and counted. Unequal sample sizes resulted when traps had blown down, or had been damaged, e.g. by cattle, bears, or people. Experimental details including objectives, beetles species, locations, dates, treatment combinations, and number of replicates are given in Table 2.3. Information on semiochemicals, purities, and sources of semiochemicals, release devices, and release rates is provided in Table 2.4. For field testing, the four stereoisomers of the putative pheromone produced by *T. betulae*, i.e. (*E*)-(3*S*,6*R*)-2-hydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol (*ESR*), (*E*)-(3*R*,6*S*)-tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol (*ERS*), (*Z*)-(3*R*,6*R*)-tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol (*ZRR*), and (*Z*)-(3*S*,6*S*)-tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol (*ZSS*), were synthesised according to the method of (Klein et al., 1964) and (Méou et al., 1991) at Simon Fraser University by

TABLE 2.3 Description of 10 field trapping experiments in BC with objectives, dates, study site and treatments listed by primary target *Trypodendron* species. All experiments comprised 10 replicates except for Experiment 1 ($N = 8$) and Experiment 5 ($N = 13$). See Table 2.4 for semiochemical information.

Species, Exp. No.	Objectives	Location and dates	Treatments
T. betulae			
1	To determine the attractiveness of the two stereoisomers of tirathol[a]. A treatment with all four stereoisomers was included.	Richmond Nature Park 25 March – 2 April 2000	1) unbaited 2) *ESR*-tirathol 3) *ZRR*-tirathol 4) *ESR*- and *ZRR*-tirathol 5) all 4 stereoisomers (*ESR*, *ZRR*, *ERS*, *ZSS*)
2	To determine if conophthorin and linalooloxide enhance the response of *T. betulae* to its pheromone.	Prince George[b] 31 March – 27 April 2001	1) unbaited 2) *ESR*- and *ZRR*-tirathol 3) *ESR* and *ZRR*-tirathol + conophthorin 4) *ESR* and *ZRR*-tirathol + linalooloxide 5) *ESR*- and *ZRR*-tirathol + conophthorin + linalooloxide
3	To test whether ethanol influences the response of *T. betulae* to *ESR*- and *ZRR*-tirathol.	Prince George 12 April – 8 May 2002	1) unbaited 2) *ESR*- and *ZRR*-tirathol 3) ethanol 4) *ESR* and *ZRR*-tirathol + ethanol
4	To test whether ethanol and conophthorin interact to increase the attractiveness of *ESR*- and *ZRR*-tirathol.	Prince George 12 April – 8 May 2002	1) unbaited 2) *ESR* and *ZRR*-tirathol + conophthorin 3) *ESR* and *ZRR*-tirathol + conophthorin + ethanol
Coastal *T. retusum, T. lineatum*			
5	To determine if the angiosperm host volatile salicylaldehyde enhances or reduces the response to lineatin of aspen-infesting *T. retusum* and conifer-infesting *T. lineatum*.	Maple Ridge[d] 27 April – 25 May 1999	1) unbaited 2) lineatin 3) salicylaldehyde 4) lineatin + salicylaldehyde

Interior *T. retusum*

6	To determine if the angiosperm host volatile salicylaldehyde enhances the response to lineatin by aspen-infesting *T. retusum*.	Princeton 17-28 April 2000	1) unbaited 2) lineatin 3) salicylaldehyde 4) lineatin + salicylaldehyde

Interior *T. retusum*

7	To compare responses by *T. retusum* to conophthorin released at a high rate (as in non-host birch) *versus* a low rate (as in its host, aspen), when combined with the host aspen volatile salicylaldehyde, and the aggregation pheromone lineatin.	Princeton[c] 28 April – 18 May 2000	1) unbaited 2) lineatin + salicylaldehyde 3) lineatin + salicylaldehyde + conophthorin (low) 4) lineatin + salicylaldehyde + conophthorin (high)
8	To determine if the response to lineatin plus salicylaldehyde by the aspen-infesting *T. retusum* is inhibited by the conifer volatile, α-pinene.	Princeton 19-27 April 2001	1) unbaited 2) lineatin + salicylaldehyde 3) lineatin + α-pinene 4) lineatin + salicylaldehyde + α-pinene
9	To determine if antennally-active host volatiles in different functional groups affect the attraction of *T. retusum* to a combination of lineatin and salicylaldehyde.	Princeton 27 April – 9 June 2001	1) unbaited 2) lineatin + salicylaldehyde 3) lineatin + salicylaldehyde + benzylalcohol + hexanol 4) lineatin + salicylaldehyde + benzyaldehyde + nonanal 5) lineatin + salicylaldehyde + linalooloxide 6) all 7 compounds
10	To test whether ethanol increases attraction of *T. retusum* to a combination of lineatin and salicylaldehyde and decreases the repellent effect of salicylaldehyde on the conifer-infesting *T. lineatum* and *T. rufitarsus*.	Princeton 20 – 27 April 2001	1) unbaited 2) lineatin 3) lineatin + salicylaldehyde 4) lineatin + ethanol 3) lineatin + salicylaldehyde + ethanol

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[a] The putative pheromone comprises the antenally-active stereoisomers *ESR*- and *ZRR*-tirathol; the *ERS* and *ZSS* stereoisomers were not detected by *T. betulae* antennae.

[b] Campus of the University of Northern British Columbia.

[c] Deep Gulch Forest Service Road.

[d] Malcolm Knapp Research Forest, University of British Columbia.

TABLE 2.4 Optical nature, purity, release devices and rates for semiochemicals used in all field trapping experiments.

Volatile compound [a, b]	Purity	Release device [c]	Release rate (mg/24h) and temperature
α-pinene [75% (-) enantiomer]	>99%	15 mL PE bottle	1.5 at 20°C
benzyl alcohol	99%	15 mL PE bubblecap	5 at 20°C
benzaldehyde	>99%	PVC flexlure	3.5 at 20°C
conophthorin[d]	92%	0.40 mL PE vial 0.25 mL PE vial	3.0 at 28°C 0.3 at 28°C
ethanol	95%	40 cm PVC pouch	40 at 20°C
hexanol	99%	15 mL PE bubblecap	2.5 at 20°C
linalooloxide (51.5% *cis*)	96.2%	0.40 mL PE vial	1.5 at 20°C[f]
lineatin	99%	PVC flexlure	0.075 at 30°C
nonanal	98%	PVC flexlure	3.5 at 20°C
salicylaldehyde	97%	15 ml PE bubblecap	5.0 at 20°C
ESR-tirathol[e]	97.6%	0.05-0.06 g LD bottle	0.11 at 20°C
ZRR-tirathol[e]	94.7%	0.05-0.06 g LD bottle	0.86 at 20°C
ERS-tirathol[e]	99.7%	0.05-0.06 g LD bottle	assumed similar to *ESR*
ZSS-tirathol[e]	95.9%	0.05-0.06 g LD bottle	assumed similar to *ZRR*

[a] All compounds were obtained from Phero Tech Inc. 7572 Progress Way, Delta, B.C. V4G1E9, unless otherwise indicated. Release rates determined at Phero Tech Inc. except for linalooloxide, the four stereoisomers of tirathol determined by Regine Gries, and conophthorin determined by D. P. W. Huber, Department of Biological Sciences, Simon Fraser University.

[b] All compounds were racemic unless otherwise specified except for tirathol. I.U.P.A.C. names if different than trivial name follow. Conophthorin, (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane; α-pinene, 2,6,6-trimethylbicyclo [3.1.1]hept-2-ene; salicylaldehyde, 2-hydroxybenzaldehyde; lineatin, 3,7-trimethyl-2,9-dioxatricyclo nonane.

[c] Release device materials: PE, polyethylene; PVC, polyvinyl chloride; LD, low-density polyethylene. All release devices were sealed except LD bottles with *ESR*, *ZRR*, *ERS*, *ZSS*, which were open and covered with an inverted, plastic, photographic film container to exclude rain.

[d] Synthesized by Drs. H.D. Pierce, Jr. and E. Czyzewska, Departments of Biological Sciences and Chemistry, respectively, Simon Fraser University, Burnaby, British Columbia, V5A 1S6.

[e] Synthesized by G. G. S. King, Jr. and E. Czyzewska, Department of Chemistry, Simon Fraser University, and H.D. Pierce Jr., Department of Biological Sciences, Simon Fraser University.

E. Czyzewska and G.G.S. King, Department of Chemistry, and H.D. Pierce, Jr., Department of Biological Sciences.[1]

Except for ethanol and *alpha*-pinene, the choice of semiochemicals was based on antennally-active compounds identified by GC-EAD analysis in captured host or beetle-emitted volatiles. Ethanol was included in some experiments as an indicator of stressed or dying trees, and *alpha*-pinene was used as a representative indicator of conifer odour.

2.3.4 Statistical Analysis

To improve normality and heteroscedasticity, all catches were transformed by $\log_{10}(x+1)$ (Zar, 1984). In all cases, this reduced the difference between the largest and smallest treatment standard deviations to less than five-fold, which is considered suitable for analysis of variance (S.A. Campbell, Department of Biological Sciences, Simon Fraser University, pers. comm.). The responses of male and female beetles were analyzed separately by ANOVA (PROC GLM, SAS v.6.0) followed by comparison of means by the Ryan-Einot-Gabriel-Welsch multiple range-test (REGWQ procedure, SAS Institute Inc., 1988; (Day and Quinn, 1989)). Experiments with replicates at different times (but the same trap locations) tested negative for a treatment × time interaction. All analyses considered block as a random effect, used Type III sums of squares, and experimentwise $\alpha = 0.05$.

[1] *ZSS* was identified by Sasaerila et al. (2003) as a sex pheromone component produced by male oil palm bunch moths, *Tirathiba mundella* Walker (Lepidoptera: Pyralidae), and given the trivial name tirathol (Sasaerila 2000). This first demonstration of pheromonal activity gives precedence to triathol as a trivial name, hence its use from this point on in my dissertation.

2.4 Results

ANOVA statistics for all field trapping experiments are listed in Table 2.5.

2.4.1 Semiochemicals for *Trypodendron betulae*

GC-EAD analysis of female and male antennal responses to captured volatiles from *T. betulae* of both sexes excised from logs (Figure 2.1, Table 2.6) revealed four EAD-active compounds, which were identified as **1** conophthorin, **2** linalooloxide, **3** nonanal, and **4** tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol (tirathol). All but **4** were also present in captured volatiles from males (Figure 2.1). The strongest response by antennae of both sexes was to tirathol, despite the relatively small amount. The next most prominent antennal responses occurred to linalooloxide. The production of the *ESR*- and *ZRR*-tirathol by female *T. betulae* (Figure 2.2) could be determined from the captured volatiles, because the elution order on a CyclodexB column of all four stereoisomeres had previously been reported (Borg-Karlson et al., 1996). The mass spectra of the isolated *ESR* and *ZRR* stereoisomers of tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol ($C_{10}H_{18}O_2$, MW:170) were consistent with those of (Méou et al., 1991) (Figure 2.3). The molecular ion with a mass over charge (m/z) value of 171 could not be detected and may be unstable under the experimental conditions used. However, other characteristic fragmentation ions could be identified. The fragmentation ion with a m/z value of 153 can be attributed to loss of water from the parent compound at C_3 leading to a fragment with the formula $C_{10}H_{16}O^+$. The fragmentation ions with a m/z values of 67 and 59 likely correspond to fragments with the formulae $C_5H_7^+$ ($H_2C{=}CH\ \ C(H_2C^+){=}CH_2$) and $C_3H_7O^+$ ($HO^+{=}C\ \ C(CH_3)_2$), respectively. Demethylation and rearrangement of latter fragment

TABLE 2.5 ANOVA results of all field trapping experiments listed by experiment number.

				MALES			FEMALES		
Exp.	**Species**	**Source of variation**	***df***	**MS**	***F***	***P***	**MS**	***F***	***P***
1	*T. betulae*	Treatment	4	0.17	3.96	0.01	0.18	3.47	0.02
		Error	28	0.04			0.05		
2	*T. betulae*	Treatment	4	0.47	3.97	0.009	0.06	5.32	0.002
		Error	36	0.12			0.04		
3	*T. betulae*	Treatment	3	0.11	1.8	0.17	0.10	2.45	0.09
		Error	27	0.20			0.24		
4	*T. betulae*	Treatment	2	1.42	9.53	0.002	1.31	13.76	0.0002
		Error	18	0.15			0.09		
5	*T. retusum*	Treatment	2	0.08	26.32	< 0.0001	1.11	16.71	< 0.0001
		Error	27	2.20			0.07		
	T. lineatum	Treatment	3	2.49	23.97	< 0.0001	1.5	14.77	< 0.0001
		Error	27	0.10			0.10		
6	*T. retusum* [a]	Treatment	3	1.11	53.4	< 0.001			
		Error	36	0.02					
	T. lineatum [a]	Treatment	3	8.74	56.98	< 0.0001			
		Error	33	0.15					
7	*T. retusum*	Treatment	3	1.68	6.46	0.002	0.3	1.24	0.32
		Error	26	0.26			0.24		
	T. lineatum	Treatment	3	4.99	16.87	< 0.0001	2.87	9.44	0.0002
		Error	26	0.29			0.30		
	T. rufitarsus	Treatment	3	0.87	0.87	0.03	0.73	3.23	0.04
		Error	26	0.25			0.22		
8	*T. retusum*	Treatment	3	4.02	39.0	< 0.0001	2.83	3.97	0.02
		Error	25	0.10			0.72		
	T. lineatum	Treatment	3	7.74	99.36	< 0.001	6.80	108.46	< 0.0001
		Error	27	0.08			0.06		
	T. rufitarsus	Treatment	3	5.28	42.98	< 0.0001	6.28	12.74	< 0.0001
		Error	26	0.12			0.49		
9	*T. retusum*	Treatment	5	1.37	23.93	< 0.0001	0.48	8.08	< 0.0001
		Error	45	0.06			0.06		
	T. lineatum	Treatment	5	4.09	70.55	< 0.0001	3.48	63.71	< 0.0001
		Error	45	0.06			0.05		
10	*T. retusum*	Treatment	4	3.28	8.89	< 0.0001	2.39	2.28	0.08
		Error	34	0.37			1.05		
	T. lineatum	Treatment	4	10.21	91.24	< 0.0001	10.56	17.25	< 0.0001
		Error	35	0.11			0.61		
	T. rufitarsus	Treatment	4	6.79	34.72	< 0.0001	7.30	10.60	< 0.0001
		Error	35	0.20			0.69		

[a] In Experiment 5, females and males were analyzed together.

Figure 2.1. Flame ionization detector (FID) analysis of Porapak Q captured volatiles from log-excised female and male *Trypodendron betulae* and electroantennographic detector (EAD) response to volatiles from females by female and male *T. betulae* antennae, chromatographed on DB-5 (0.32 ID) column, temperature program: 1 min at 50°C rising at 10°C /min to 280°C, splitless injection. EAD-active compounds 1-4 were identified as follows: (**1**) = conophthorin (retention index 6.04), (**2**) = linalooloxide (retention index 6.55), (**3**) = nonanal (retention index 6.78), and (**4**) = tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol (tirathol) (retention index 7.82).

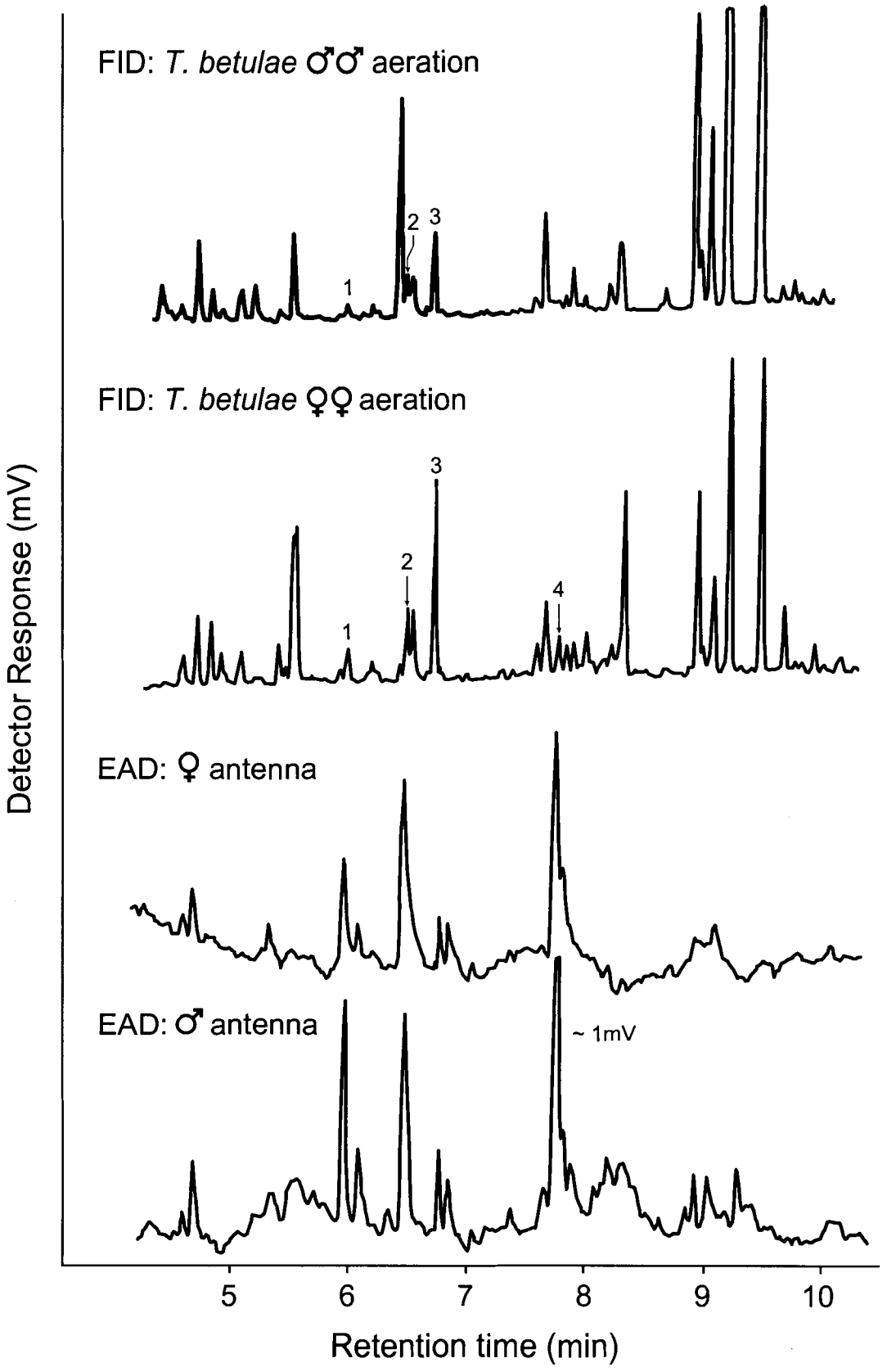

FID: T. betulae ♂♂ aeration
1
2
3
FID: T. betulae ♀♀ aeration
1
2
3
4
EAD: ♀ antenna
EAD: ♂ antenna
~ 1mV
Detector Response (mV)
5
6
7
8
9
10
Retention time (min)

TABLE 2.6 Antennal responses (+) of female and male *Trypodendron* of North American and European origin to host, non-host and/or beetle produced volatiles. Dashes denote no response. Blanks indicate compounds not tested.

	North America										Europe			
	T. lineatum		*T. rufitarsus*		*T. retusum*		*T. betulae*		*T. domesticum*		*T. domesticum*		*T. signatum*	
Compound														
(+)-lineatin	+	+	+	+	+	+			+	+	+	+	+	+
(-)-lineatin														
ESR-tirathol							+	+	+	+	+	+	+	+
ZRR-tirathol							+	+						
ERS-tirathol														
ZSS-tirathol														
α-pinene	+	+	+	+	+	+	+	+			+	+	+	+
benzyl alcohol	+	+			+	+								
benzaldehyde					+	+					+	+		
conophthorin	+	+	+	+	+	+	+	+					+[a]	
ethanol														
hexanol	+	+			+	+					+	+	+	+
linalooloxide					+	+	+	+						
nonanal	+	+			+		+	+			+	+		
salicylaldehyde	+	+	+	+	+	+					+	+	+	+

[a] response to (-)-conophthorin only.

Figure 2.2 FID analysis of four stereoisomers of tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol (*ERS*, *ESR*, *ZSS*, *ZRR*, respectively) on a CyclodexB chiral column fitted with a splitless injector and run with a temperature program of 1 min at 80°C rising at 5°C /min to 120°C isothermal, with a corresponding analysis of captured volatiles from *T. betulae* females showing natural production only of *ESR* and *ZRR* stereoisomers.

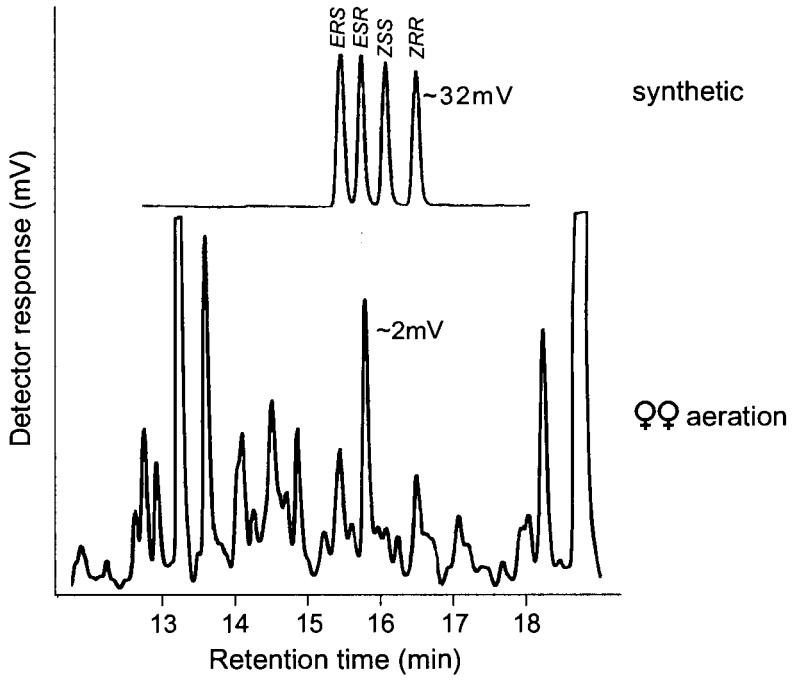
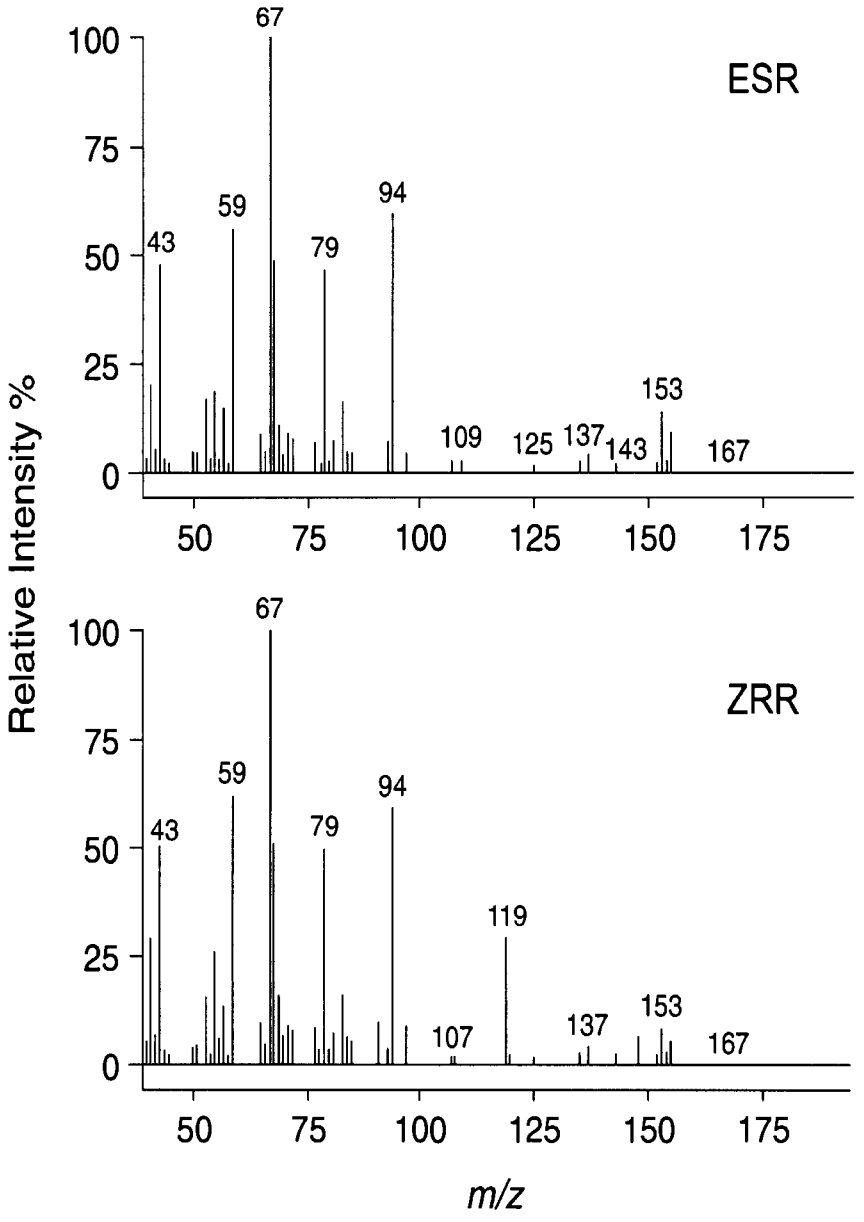

ERS
ESR
ZSS
ZRR
~32mV
synthetic
~2mV
♀♀ aeration
Detector response (mV)
13
14
15
16
17
18
Retention time (min)

Figure 2.3. Mass spectra of *ESR*- and *ZRR*-tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol isolated from captured volatiles produced by female *T. betulae*.


ESR
ZRR
Relative Intensity %
m/z
100
75
50
25
0
50
75
100
125
150
175
67
59
43
79
94
109
125
137
143
153
167
119
107

likely gave rise to the fragment with a m/z value of 43, i.e. $C_2H_3O^+$ (O^+ C CH_3). When all four stereoisomers of synthetic tirathol were analysed by GC-EAD with a chiral column (Figure 2.4), antennae of both sexes responded only to the *ESR* and *ZRR* stereoisomers. These results suggest that the two stereoisomers together constituted a promising candidate pheromone.

Catches of female *T. betulae* in Experiment 1 were significantly higher in multiple-funnel traps baited with *ESR* and *ZRR* tirathol in combination (Table 2.5, Figure 2.5) than with either stereoisomer alone or all four together. This trend was reflected in catches of males, but a significant difference only occurred between catches in traps baited with *ESR* and *ZRR* tirathol and those in unbaited traps or traps baited with only the *ESR* stereoisomer.

Analysis of paper birch bark volatiles by GC-EAD disclosed five antennally-active compounds in both sexes of *T. betulae* (Figure 2.6). Only three of the five compounds were identified: **1** conophthorin, **2** unknown, **3** nonanal, **4** methyl-eugenol, and **5** unknown. The strong antennal response to conophthorin, and it prevalence in the bark volatiles of paper birch (Huber et al., 1999) suggested that it could be an attractive host kairomone. When conophthorin and linalooloxide were added alone or together to tirathol a differential response occurred (Figure 2.7; Experiment 2). The response by females was similar to that in Experiment 1 (Figure 2.5), with catches in traps baited with *ESR*- and *ZRR*-tirathol higher than in unbaited traps, regardless of the presence of conophthorin. For males, however, a higher catch than in unbaited traps occurred only when conophthorin was present. In contrast, when linalooloxide was added to *ESR*- and *ZRR*-tirathol and conophthorin it had a repellent effect in both sexes (Figure 2.7;

Figure 2.4. Flame ionization detector (FID) response (top) to the four synthetic stereoisomers of tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol (*ERS*, *ESR*, *ZSS*, *ZRR)*, their chemical structures, and (bottom) electroantennographic detector (EAD) responses by female and male *Trypodendron betulae* antennae showing detection of only the *ESR* and *ZRR* stereoisomers. Chromatography: CyclodexB chiral column, temperature program: 1 min at 110°C, rising at 5°C/min to 120°C, splitless injection.

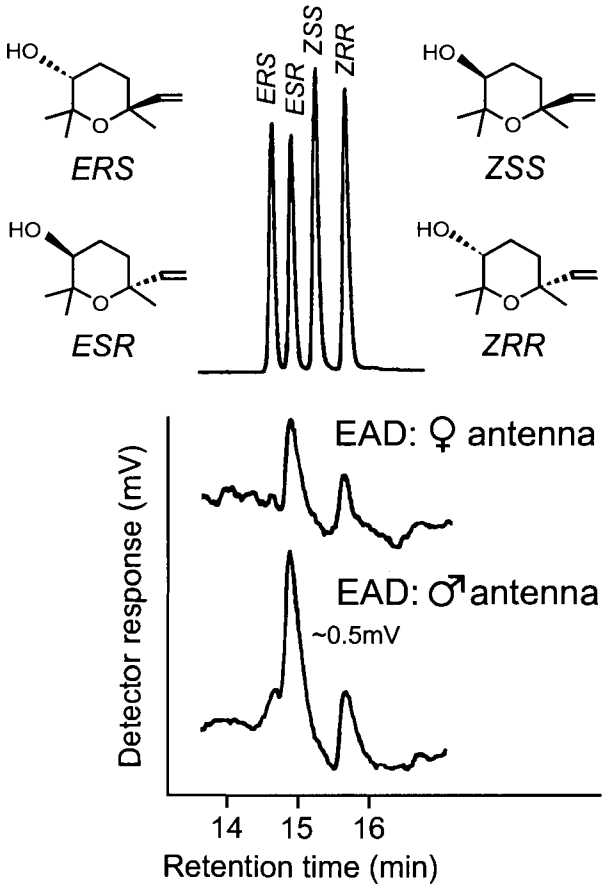

HO
ERS
HO
ESR
ERS
ESR
ZSS
ZRR
HO
ZSS
HO
ZRR
EAD: ♀ antenna
EAD: ♂ antenna
~0.5mV
Detector response (mV)
14
15
16
Retention time (min)

Figure 2.5 Numbers of *Trypodendron betulae* females and males captured in Experiment 1 in multiple-funnel traps baited with the two antennally-active steroeisomers of tirathol (*ESR* and *ZRR*) alone, together or together in combination with the antennally inactive *ERS* and *ZSS* stereoisomers. For each sex, means with the same letter are not significantly different, REGW multiple-range test, $P < 0.05$.

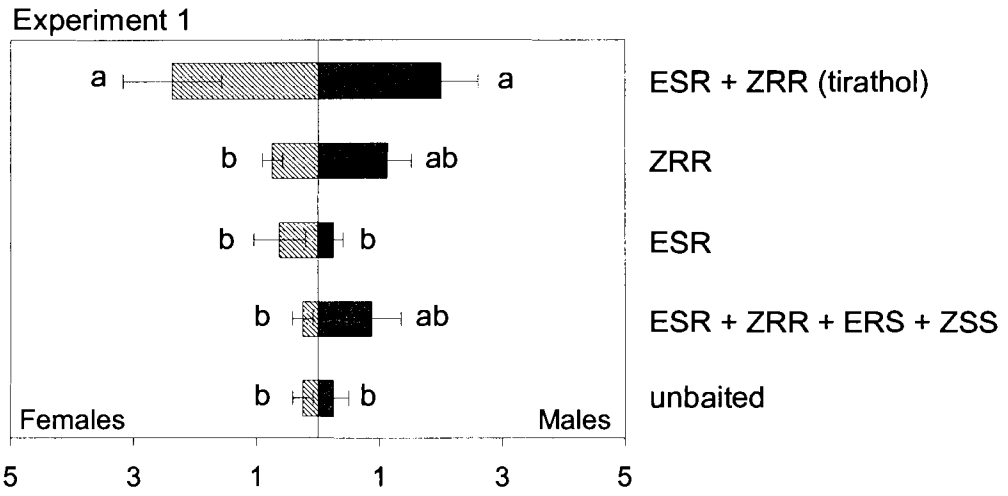

Experiment 1
a
a
b
ab
b
b
b
ab
b
b
ESR + ZRR (tirathol)
ZRR
ESR
ESR + ZRR + ERS + ZSS
unbaited
Females
Males
5
3
1
1
3
5
Number of T. betulae captured (mean ± SE)

Figure 2.6. Flame ionization detector (FID) analysis and electroantennographic detector (EAD) responses by female and male *Trypodendron betulae* antennae to volatiles captured from paper birch on Porapak Q. EAD-active compounds 1-5 were determined as follows: (**1**) = conophthorin, (**2**) = unidentified, (**3**) = nonanal, (**4**) = methyl-eugenol, and (**5**) = unidentified. Chromatography: DB-210-8 column, column fitted with a splitless injector and run at a temperature program of 1 min at 50°C rising10°C/min to 220°C.

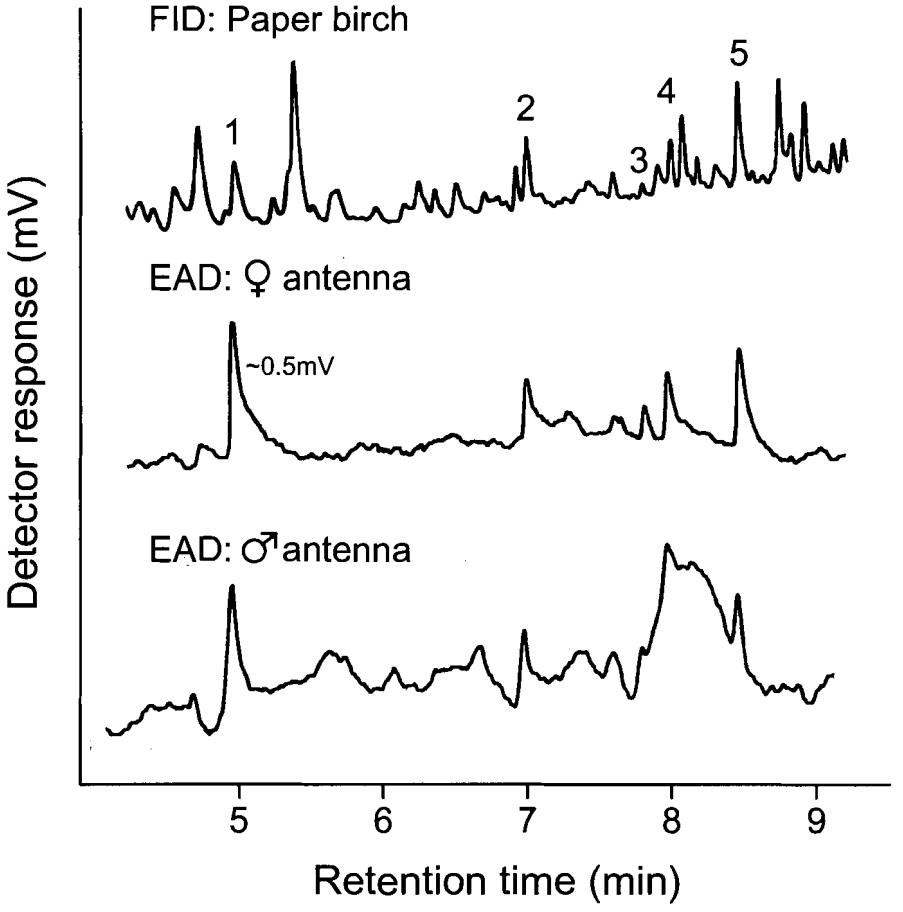
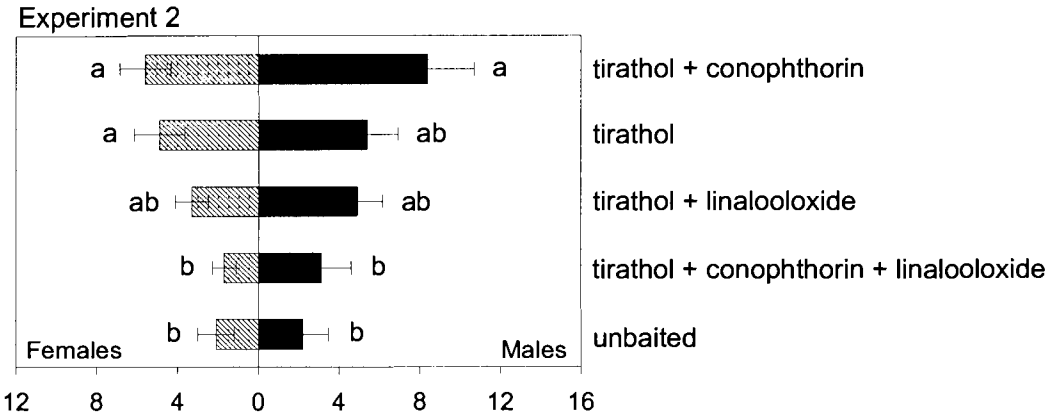
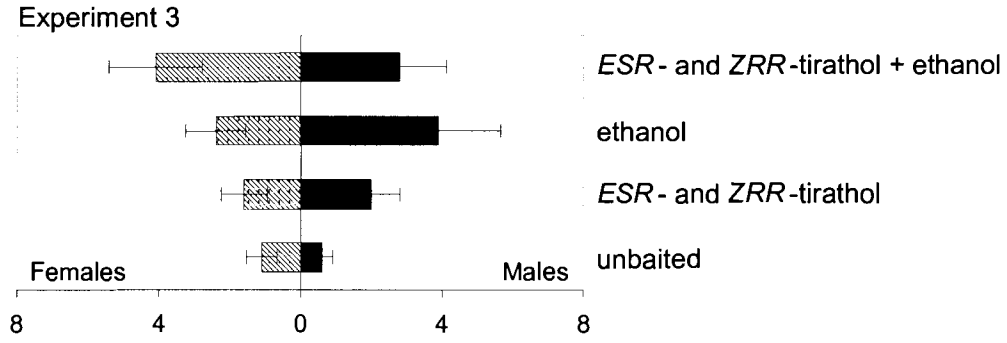
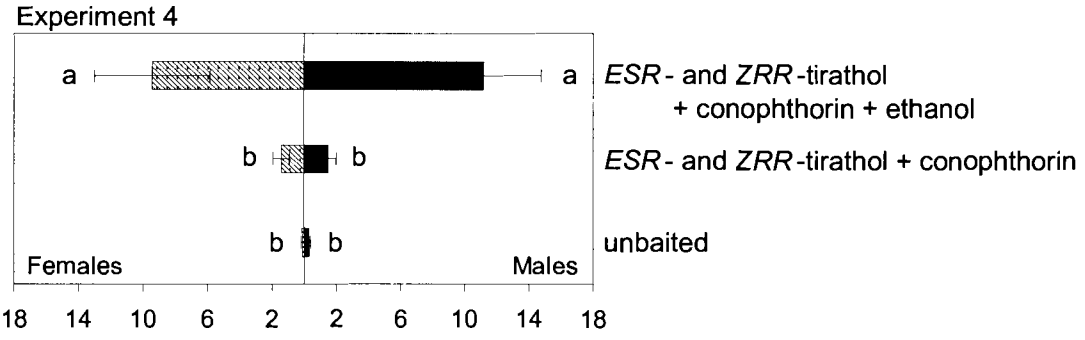

FID: Paper birch
1
2
3
4
5
EAD: ♀ antenna
~0.5mV
EAD: ♂ antenna
Detector response (mV)
5
6
7
8
9
Retention time (min)

Figure 2.7 Numbers of *Trypodendron betulae* captured in multiple-funnel traps baited with *ESR*- and *ZRR*-tirathol alone or in combination with conophthorin and linalooloxide separately or together (Experiment 2), in combination with ethanol (Experiment 3), or in combination with conophthorin and ethanol (Experiment 4). For each experiment and sex, means with the same letter are not significantly different, REGW multiple-range test, $P < 0.05$. Analysis of variance in Experiment 3 revealed no significant difference (Table 2.5).







Number of *T. betulae* captured (mean ± 1 SE)

Experiment 2). When the host kairomone ethanol was added to *ESR*- and *ZRR*-tirathol, neither sex was more attracted than when traps were baited with *ESR*- and *ZRR*-tirathol alone (Figure 2.7; Experiment 3). However, when conophthorin and ethanol were combined with tirathol, there was a strong synergistic effect for both sexes, with catches of females and males being over seven times higher than in traps baited with the blend of *ESR*- and *ZRR*-tirathol with conophthorin (Figure 2.7; Experiment 4).

2.4.2 Chemical Ecology of *Trypodendron* species

GC-EAD analysis of volatiles from *T. retusum* and *T. rufitarsus* females captured in traps revealed a strong response by antennae of both sexes of each species to a peak matching the retention time of lineatin (data not shown). For *T. retusum*, further analysis of volatiles of males and females that were excised 1-2 days after attacking standing trembling aspen trees in the field, revealed that only females that initiated the galleries had produced lineatin at a detectable level (Figure 2.8). The mass spectra of the isolated lineatin confirmed its identification (Figure 2.9). Failure to induce attack by *T. rufitarsus* on lineatin-baited lodgepole pines in 2000, and removal of lineatin-baited logs by firewood cutters in 2003 precluded analysis of volatile production by *T. rufitarsus* that had attacked new hosts.

Among eight host and non-host tree volatiles tested by GC-EAD among the four *Trypodendron* species, salicylaldehyde and conophthorin generally elicited the strongest antennal response (data not shown). In Experiment 5, coastal *T. lineatum* and *T. retusum* were differentially affected by salicylaldehyde, which inhibited response to lineatin by the former species, and synergized the response by the latter species (Figure 2.10).

Figure 2.8 Flame ionization detector (FID) analysis of volatiles obtained from male and female *Trypodendron retusum* captured in lineatin-baited traps, or excised from logs of trembling aspen indicating that only females that had bored into logs produced lineatin in detectable amounts. Chromatography: DB-5 (0.32 ID) column, temperature program: 1 min at 50°C rising at 10°C/min to 280°C.

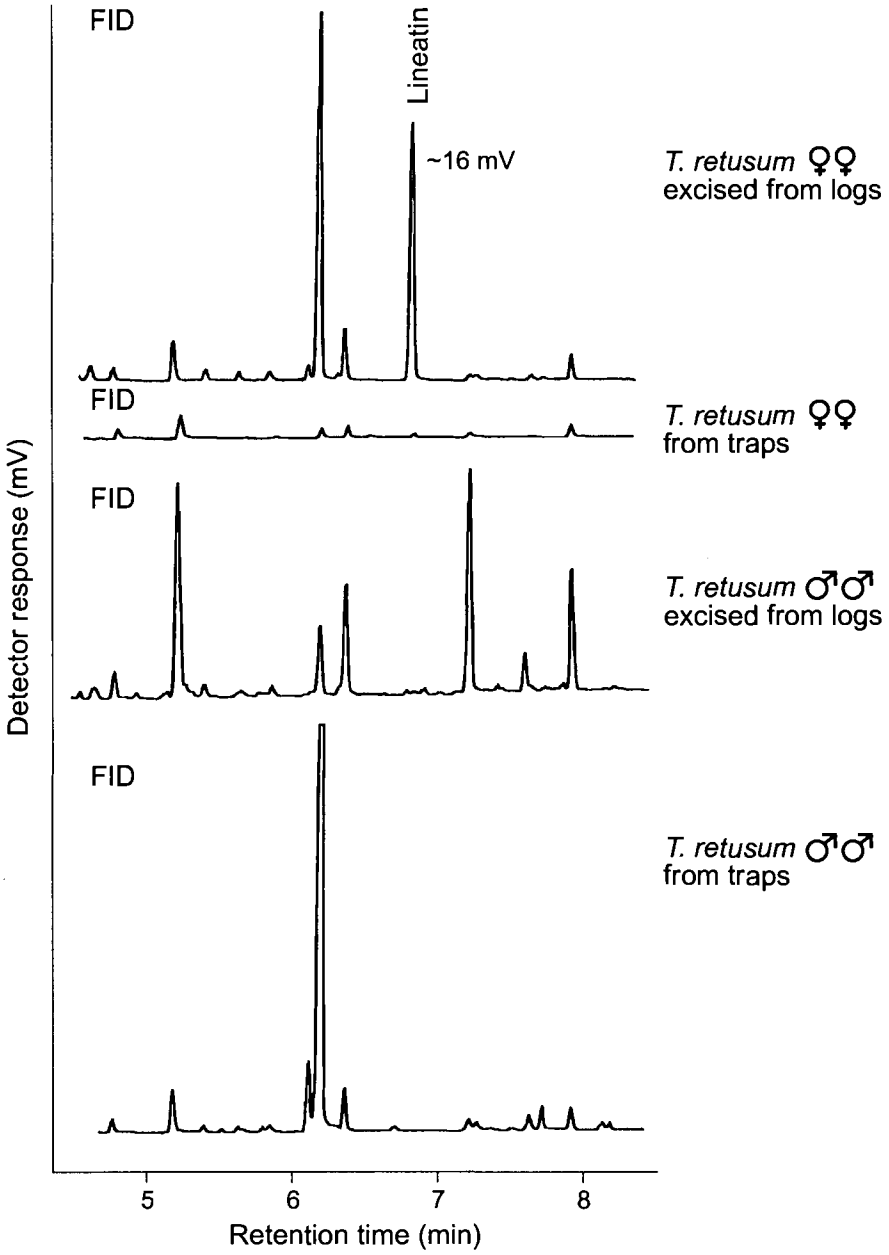

FID
Lineatin
~16 mV
T. retusum ♀♀
excised from logs
FID
T. retusum ♀♀
from traps
FID
T. retusum ♂♂
excised from logs
FID
T. retusum ♂♂
from traps
Detector response (mV)
5
6
7
8
Retention time (min)

Figure 2.9 Mass spectrum of lineatin produced by female *Trypodendron retusum* confirming production of the (+) enantiomer (MacConnell et al., 1977).

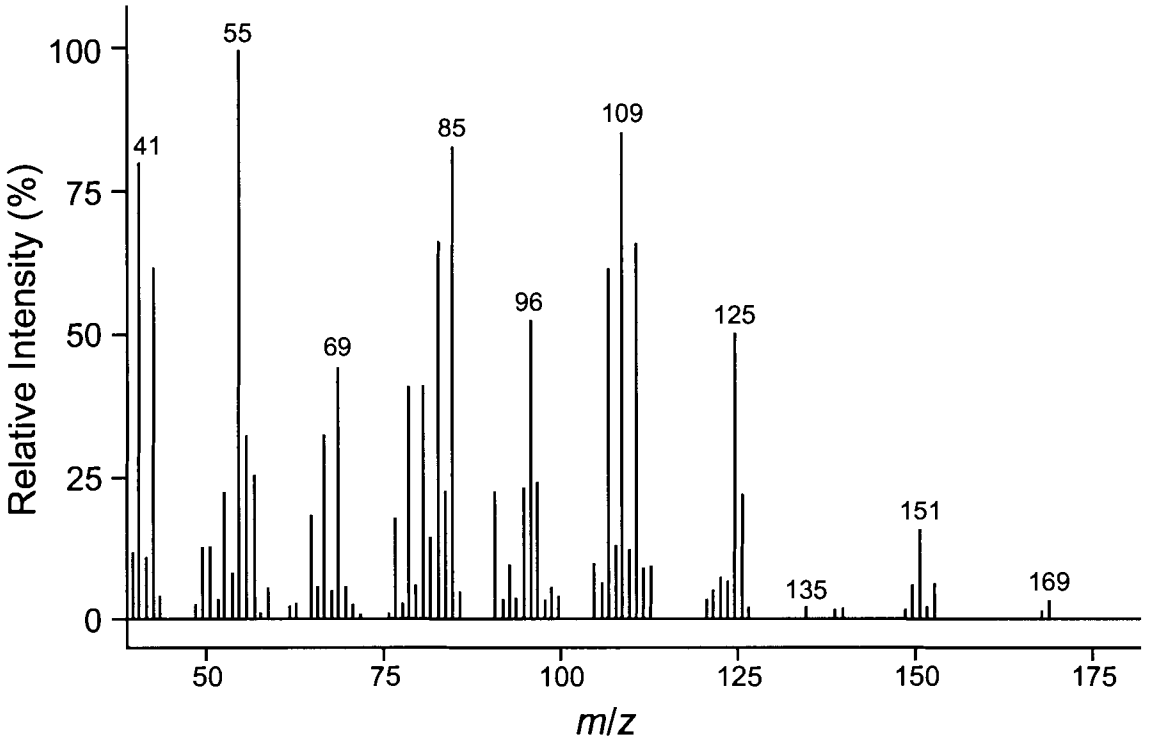
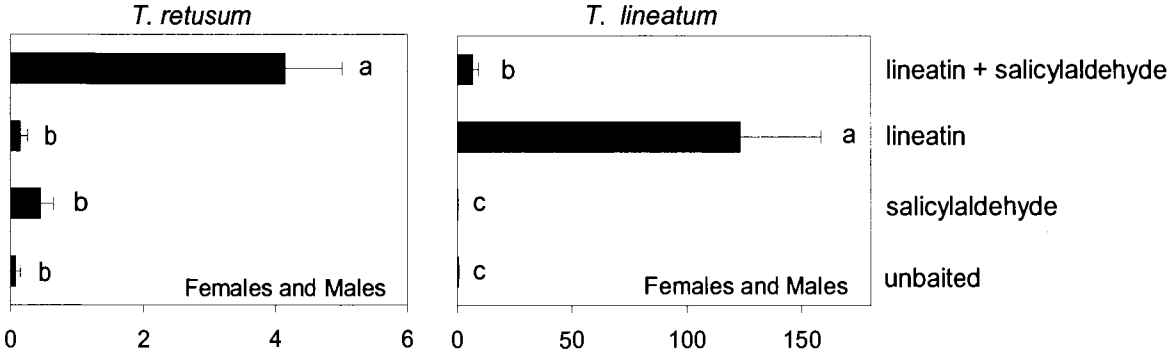
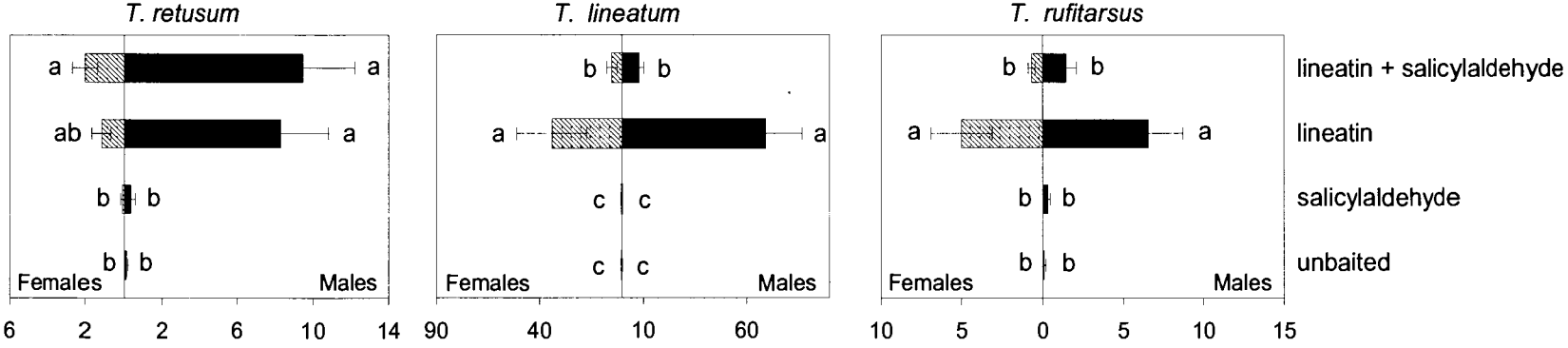

Relative Intensity (%)
100
75
50
25
0
55
41
85
109
69
96
125
151
135
169
50
75
100
125
150
175
m/z

Figure 2.10 Numbers of coastal *Trypodendron lineatum* and *T. retusum* captured in Experiment 5 (top) showing a differential effect of salicylaldehyde when combined with (±)-lineatin. Sex of beetles not determined. Numbers of interior *T. retusum*, *T. lineatum* and *T. rufitarsus* captured in Experiment 6 (bottom) showing a differential effect of salicylaldehyde on angiosperm-infesting *T. retusum*, and on two conifer-infesting species, *T. lineatum* and *T. rufitarsus*. For each experiment and sex, means with the same letter are not significantly different, REGW multiple-range test, $P < 0.05$.



Number of coastal *Trypodendron* captured in Experiment 5 (mean ± SE)



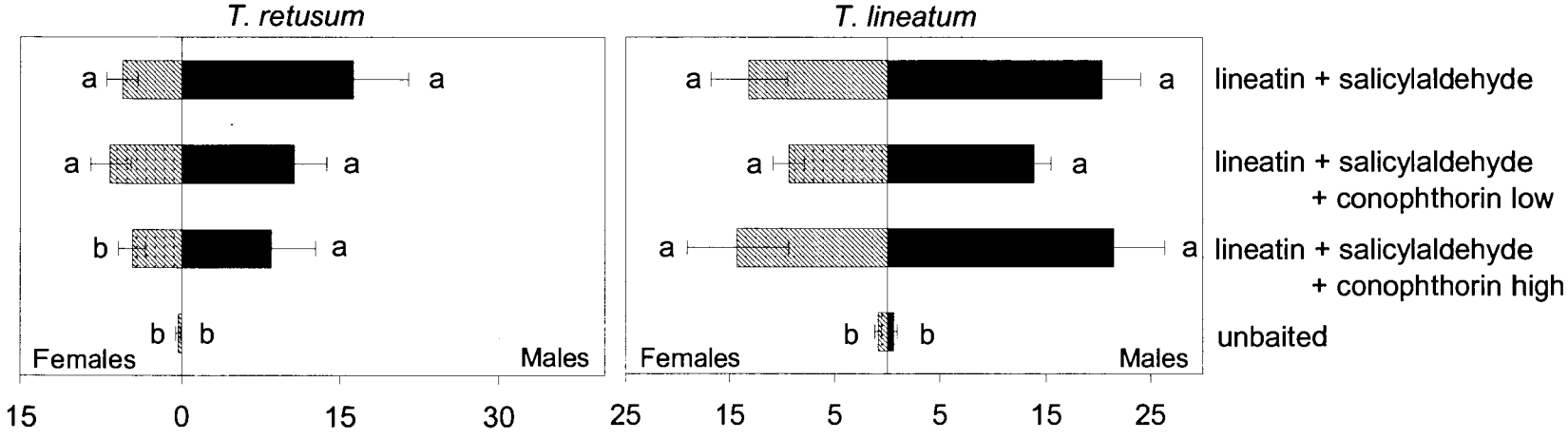
Number of interior *Trypodendron* captured in Experiment 6 (mean ± SE)

Lineatin alone was not attractive to coastal *T. retusum*, and traps baited with salicylaldehyde alone captured no more beetles of either species than unbaited control traps.

Experiments 6-10 in the interior of B. C. were originally designed to elucidate the chemical ecology of *T. retusum*. However *T. lineatum* and *T. rufitarsus* were often captured and allowed my objectives to be extended to investigate the chemical ecology of all three sympatric species. In Experiment 6, both sexes of interior *T. lineatum* and *T. rufitarsus*, both conifer-inhabiting, were repelled by salicylaldehyde (Figure 2.10) in an identical manner to coastal *T. lineatum* (Figure 2.10). Unlike coastal *T. retusum*, interior *T. retusum* did not respond in a synergistic manner to lineatin with salicylaldehyde (Figure 2.10).

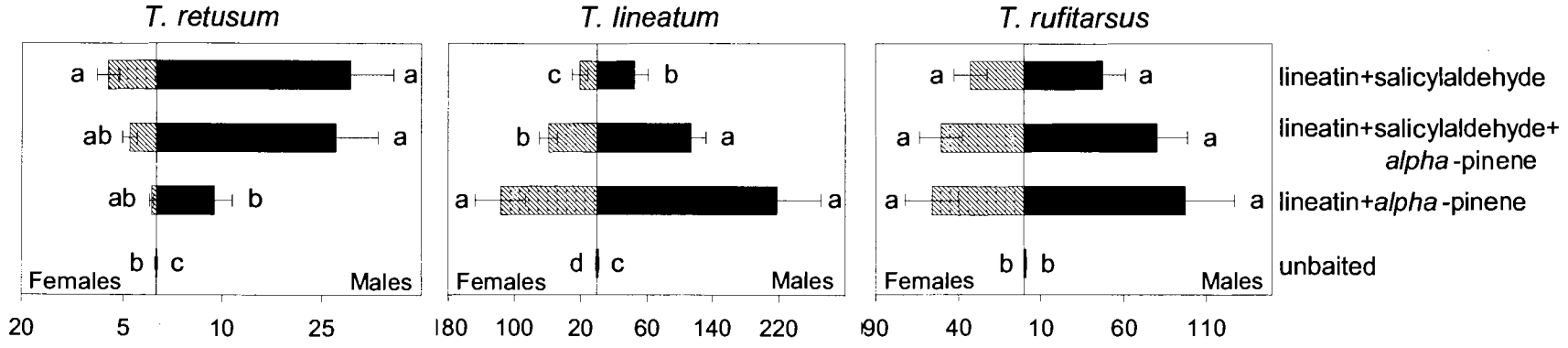
The addition of conophthorin released at a high level (simulating trembling aspen trees) or a low level (simulating paper birch trees) to the blend of lineatin and salicylaldehyde had no effect on the numbers of interior *T. retusum* or *T. lineatum* captured Experiment 7 (Figure 2.11). Addition of the conifer volatile, *alpha*-pinene, to the blend of lineatin plus salicylaldehyde did not have an inhibitory effect on the response of interior *T. retusum* in Experiment 8 (Figure 2.12). However, *alpha*-pinene did enable *T. lineatum*, but not *T. rufitarsus*, to escape the inhibitory effect of combining salicylaldehyde with lineatin. Neither linalooloxide, nor two alcohols (benzyl alcohol and 1-hexanol), nor two aldehydes (benzaldehyde and nonanal) affected the numbers of *T. retusum* captured in traps baited with lineatin plus salicylaldehyde in Experiment 9 (Figure 2.13). However, for *T. lineatum* the two aldehydes appeared to counteract the inhibitory effect of adding salicylaldehyde to lineatin, and the two alcohols enhanced the

Figure 2.11. Numbers of interior *Trypodendron retusum* and *T. lineatum* captured in Experiment 7 testing the effect of high and low release rates of conophthorin (Table 2.4) to a blend of (±)-lineatin and salicylaldehyde. For each species and sex, means with the same letter are not significantly different, REGW multiple-range test, $P < 0.05$.



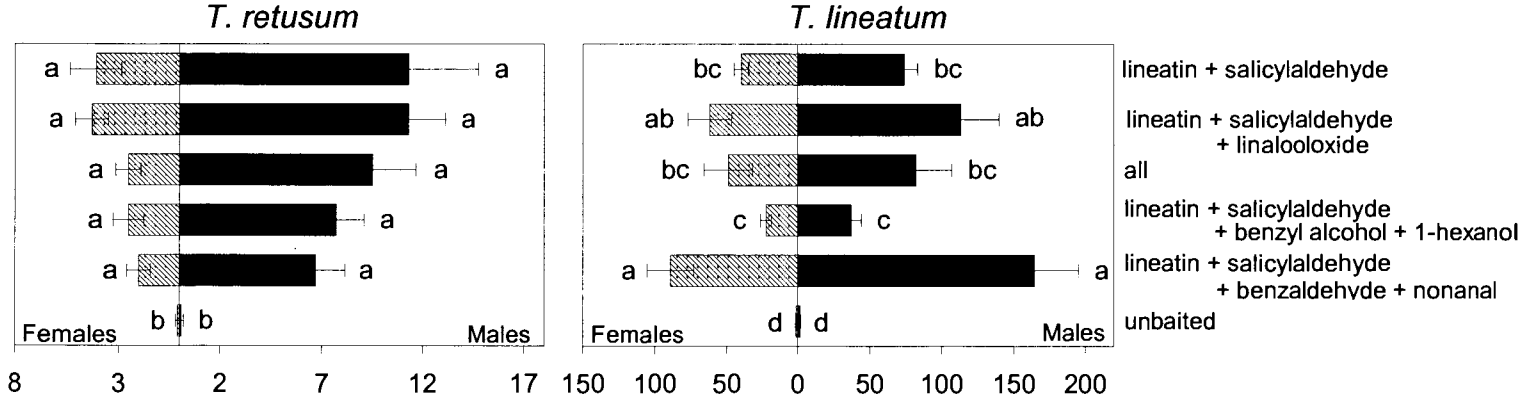
Number of *Trypodendron* captured in Experiment 7 (mean ± SE)

Figure 2.12. Numbers of three interior *Trypodendron* species captured in Experiment 8, showing a species-specific effect of combining the angiosperm volatile, salicylaldehyde, and the conifer volatile *alpha*-pinene, alone or together with the aggregation pheromone, (±)-lineatin. For each sex, means with the same letter are not significantly different, REGW multiple-range test, $P < 0.05$.



Number of interior *Trypodendron* captured in Experiment 8 (mean ± SE)

Figure 2.13. Numbers of interior *Trypodendron retusum* and *T. lineatum* captured in Experiment 9 testing the effect of adding linalooloxide, two alcohols (benzylalcohol, 1-hexanol), two aldehydes (benzaldehyde, nonanal), or all five compounds together to a blend of (±)-lineatin and salicylaldehyde. For each sex, means with the same letter are not significantly different, REGW multiple-range test, $P < 0.05$.



Number of *Trypodendron* captured in Experiment 9 (mean ± SE)

inhibition of males.

In Experiment 10, adding ethanol to the blend of lineatin and salicylaldehyde caused a synergistic effect in the number of female *T. retusum* captured, and appeared to obscure a positive response to the binary blend (Figure 2.14). Ethanol had no effect on the response of *T. lineatum* to lineatin alone or with salicylaldehyde, but for *T. rufitarsus* it partially counteracted the inhibitory effect of combining salicylaldehyde with lineatin.

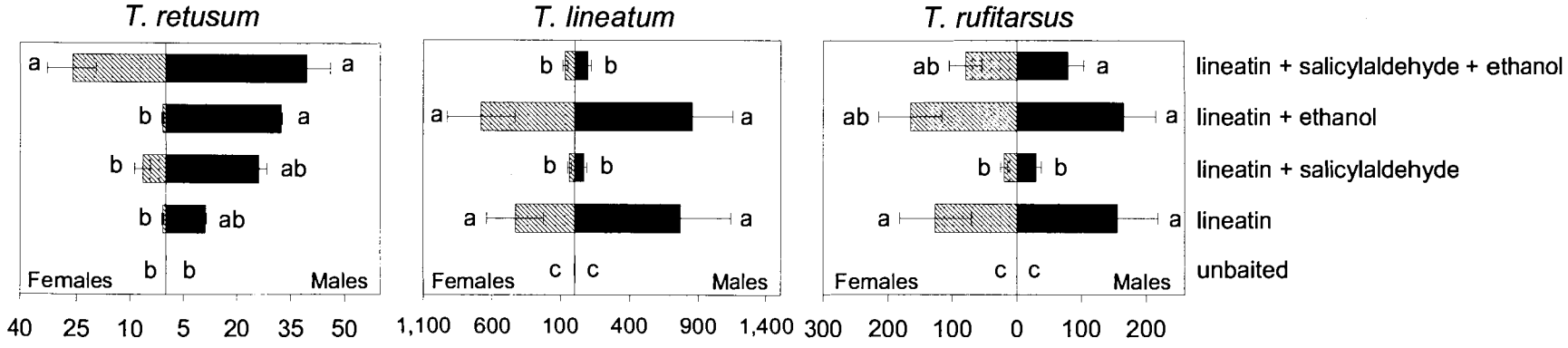
The responses to (+)- and (-)-lineatin, and all four stereoisomers of tirathol by female antennae all five sympatric species, *T. lineatum*, *T. rufitarsus*, *T. retusum*, *T. betulae* and exotic *T. domesticum* (Figure 2.15) reveal that all species but *T. betulae* detect (+)-lineatin, but not (-)-lineatin. Conversely *T. betulae* antennae detected only *ESR*- and *ZRR*-tirathol. Unexpectedly, *T. domesticum* also detected *ESR*-tirathol.

2.5 Discussion

2.5.1 Semiochemicals for *Trypodendron betulae*

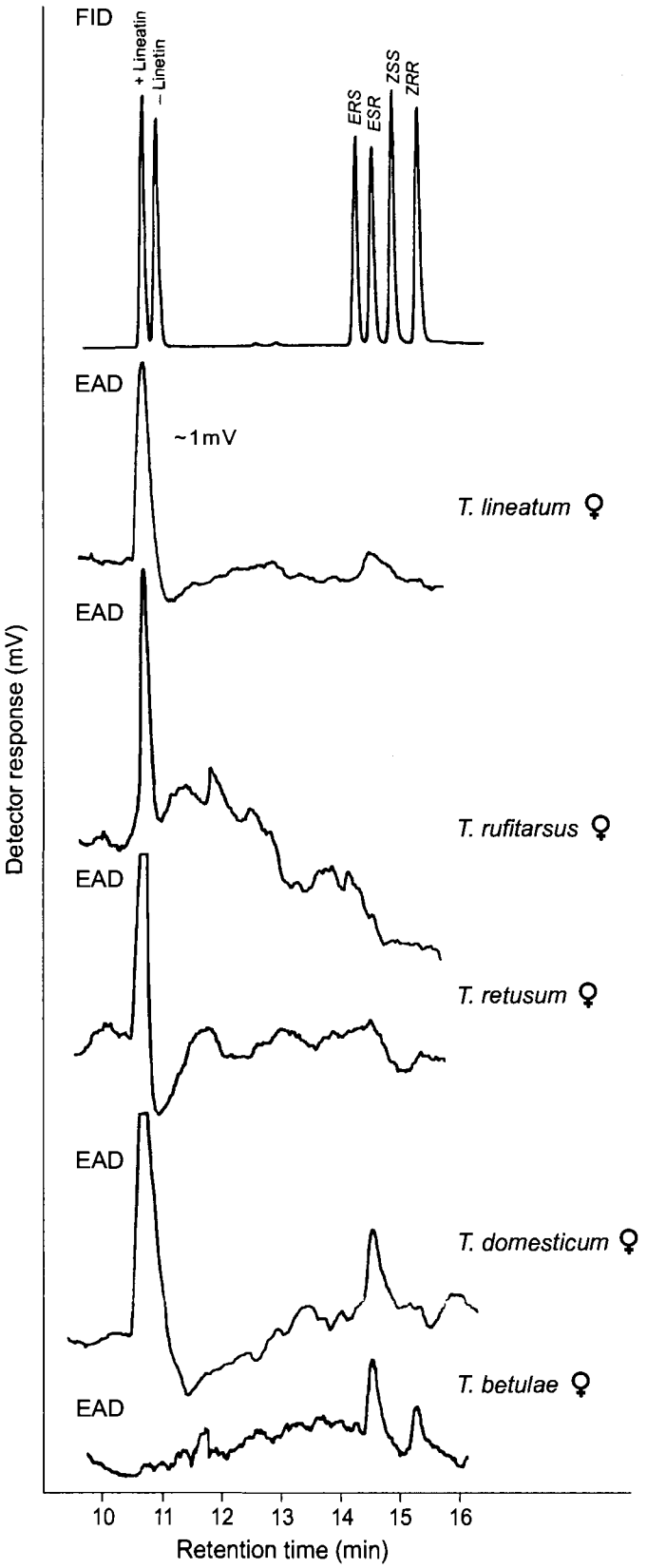
My results demonstrate that a blend of the *ESR* and *ZRR* stereoisomers of tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol (tirathol) is the female-produced aggregation pheromone of *T. betulae*. *ZSS*-tirathol was identified by Sasaerila et al. (2003) as a sex pheromone component produced by male oil palm bunch moths *Tirathaba mundella* Walker (Lepidoptera: Pyralidae). The *ZSS*- and *ZRR*- stereoisomers were isolated from the elm bark beetle, *Pteleobius vittatus* (F.) (Chikashita et al., 1993; Mori and Puapoomchareon, 1988), but their behavioural role was unclear. The *ESR*- and *ZSS*- isomers are also present in the shrub, *Daphna mezereum* L. (Tymelaeaceae) and may attract solitary bees (Borg-Karlson et al., 1996). In addition to tirathol, furanoid or pyranoid compounds were detected in these systems (Borg-Karlson et al., 1996),

Figure 2.14 Numbers of three interior *Trypodendron* species captured in Experiment 10 testing the effect of adding ethanol alone or with salicylaldehyde to the aggregation pheromone lineatin. For each sex, means with the same letter are not significantly different, REGW multiple-range test, $P < 0.05$.



Number of interior *Trypodendron* captured in Experiment 10 (mean ± SE)

Figure 2.15 Flame ionization detector (FID) and electroantennographic detector (EAD) response to synthetic (+)- and (-)-lineatin and the four stereoisomeres of tirathol by antennae of female *T. lineatum*, *T. rufitarsus*, *T. retusum*, *T. domesticum*, and *T. betulae*. Chromatography: CyclodexB chiral column, temperature program: 11.5 min at 110°C, rising at 5°C/min to 120°C, split injection.


FID
+ Lineatin
– Linetin
ERS
ESR
ZSS
ZRR
EAD
~1mV
T. lineatum ♀
EAD
T. rufitarsus ♀
EAD
T. retusum ♀
EAD
T. domesticum ♀
T. betulae ♀
EAD
Detector response (mV)
10
11
12
13
14
15
16
Retention time (min)

either of which can be used as starting compound in the synthesis of tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol (Chikashita et al., 1993; Klein et al., 1964; Mori and Puapoomchareon, 1988). In *T. betulae* linalooloxide is present in the volatiles of both sexes and is a potentially a plant-derived precursor for the production of tirathol. The slight repellent effect of linalooloxide (which is perceived by *T. betulae* antennae) when presented with *ESR*- and *ZSS*- tirathol is unexplained.

Plettner (2002) hypothesized that synthetic analogues of pheromones could be found that would interfere with olfactory responses by blocking the pheromone binding protein or the transmembrane receptor site. These "pheromone olfactory inhibitors" should meet three criteria: 1) they should not be "attractive, repellent or antagonistic" by themselves; 2) they should be "volatile and active at concentrations similar to the pheromone"; and 3) they "would be designed to inhibit selected components of the peripheral olfactory system, thereby significantly increasing the insect's response threshold to the pheromone." Trifluoromethylketone and thiotrifluoropropanone analogs of lepidopteran pheromones have been found in electrophysiological studies to not cause dipolarization of olfactory receptors, but to decrease depolarization and spike frequency generated by olfactory neurons when offered with the insect's sex pheromone (Pophof, 1998; Pophof et al., 2000). However, no behavioural evidence has been found to date for the bioactivity of pheromone olfaction inhibitors. *ERS*- and *ZSS*-tirathol meet all three of the above criteria, and when offered with the pheromone.

ERS- and *ZSS*-tirathol significantly reduced the catches of female *T. betulae* and reduced the catches of males to a level not significantly higher than in unbaited control traps (Figure 2.5). This appears to be the first authenticated example of the bioactivity of

pheromone olfaction inhibitors based on a combination of olfactory and behavioural evidence.

Linalooloxide and conophthorin were chosen for field-testing over nonanal, because they produced stronger antennal responses in both sexes (Figure 2.1). Conophthorin is not only emitted by *T. betulae* females and males, but also constitutes 12% of bark volatiles of paper birch (Huber et al., 1999). Conophthorin is also a male-produced antiaggregation pheromone in cone beetles, *Conophthorus* spp., the ash bark beetle, *Leperisinus varius* (F.), and the fir bark beetle, *Cryphalus piceae* (Ratzeburg). (Birgersson et al., 1995; Dallara et al., (1994); Kohnle et al., 1992; Pierce et al., 1995); and also a possible aggression inhibitor in the wasp, *Paravespula vulgaris* L. (Francke et al., 1978). In *T. betulae* conophthorin apparently acts both as a female-produced aggregation pheromone and as an attractive host kairomone. However, it is not known whether it is sequestered by females from host plant tissue, or synthesized *de novo*. Neither conophthorin nor ethanol, a ubiquitous kairomonal indicator of stressed hosts undergoing anaerobic metabolism (Kelsey, 1994), had a pronounced effect on trap catches when added individually to tirathol. However, when combined, conophthorin and ethanol acted synergistically with *ESR*- and *ZRR*-tirathol (Figure 2.7). This blend of semiochemicals, with different origins and individual functions, conveys an apparently unique species-specific message used by *T. betulae* to locate hosts and attract mates.

2.5.2 Chemical Ecology of Five *Trypodendron* species

My results show for the first time that female *T. retusum* produce (+)-lineatin, that it is absent in males, and that antennae of both sexes perceive only (+)-lineatin. These results provide a mechanistic explanation for the finding that *T. retusum* is attracted to

traps baited with (+)-lineatin but not (-)-lineatin, while there is no inhibitory effect of (-)-lineatin (Hoover et al., 2000; Lindgren et al., 2000).

The differential role of the angiosperm volatile salicylaldehyde as a kairomone for coastal but not interior *T. retusum* (Figure 2.10) may be contingent on the prevalence of the available host species. In coastal habitats *Populus* spp. occur rarely in forests dominated by conifers. Thus it would be adaptive for coastal *T. retusum* to respond synergistically to the combination of lineatin and salicylaldehyde during the host selection phase. In contrast large groves of trembling aspen occur in the B.C. interior and a synergistic interaction between lineatin, salicylaldehyde and ethanol (Figure 2.14), rather then between lineatin and salicylaldehyde, would facilitate attack on suitable, stressed hosts scattered among healthy trees. Such a phylogeographic differentiation between ecotypes of *T. retusum*, are analogous to the different responses to attractive semiochemicals by *T. lineatum* in B.C. and the United Kingdom (Borden et al., 1982), or to semiochemical-based differences between two genetically distinct populations of the white pine weevil, *Pissodes strobi* Peck, with coastal females preferring host volatiles and interior females orienting to male-produced pheromone (Tanaka, 2003).

The reliance on a single pheromone, (+)-lineatin, by three native sympatric *Trypodendron* species in B.C. (Figure 2.15) is remarkable. Only *T. betulae* employs a different pheromone, *ESR*- and *ZRR*-tirathol. Therefore species-specificity in chemical communication among *T. lineatum*, *T. rufitarsus*, and *T. retusum* must rely on other signals, e.g. host and non-host kairomones, and possibly as yet undiscovered synomones.

Tirathol (Figure 2.4) is chemically very different from (+)-lineatin and *T. betulae* is semiochemically distinct from all other species in the genus. *Trypodendron betulae* did

not respond to either (+)- or (-)-lineatin in GC-EAD analysis (Figure 2.15) nor in field bioassays (Hoover et al., 2000; Lindgren et al., 2000). Moreover, all other native *Trypodendron* spp. did not detect any stereoisomer of tirathol, nor were they attracted to traps baited with *ESR*- and *ZRR*-tirathol. Therefore neither pheromone can function as a repellent synomone, as occurs among sympatric species of moths (Evenden et al., 1999; Linn and Roelofs, 1995). Because of the species-specificity of *ESR*- and *ZRR*-tirathol, *T. betulae* would not have experienced selective pressure to evolve the ability to recognize (-)-lineatin as a reinforcing synomonal repellent.

The established and exotic *T. domesticum* responded antennally to both (+)-lineatin and *ESR*-tirathol. In one preliminary field experiment *T. domesticum* responded to traps that were baited with only *ESR*- and *ZRR*-tirathol, while in another experiment the response to traps baited with lineatin was strong, while only a few beetles were attracted to *ESR*- and *ZRR*-tirathol, and intermediate numbers flew to traps baited with lineatin and tirathol. The role of *ESR*-tirathol in the chemical ecology of *T. domesticum* thus remains unclear, but these signalling characteristics could maintain partial reproductive isolation of the newly sympatric exotic *T. domesticum* in B.C. All *Trypodendron* species in Europe that are sympatric with *T. domesticum* also produce only (+)-lineatin and respond behaviourally to (±)-lineatin (Klimetzek et al., 1981; Schurig et al., 1982).

The integration of host kairomones with insect-produced pheromones may shape semiochemical signals that function in behavioural reproductive isolation of sibling species (Landolt and Phillips, 1997; Miller and Borden, 1990a; Miller and Borden, 2000; Pitman, 1969; Seybold et al., 1995; Wood, 1982a). Separation of the angiosperm

infesting *T. retusum*, and the coniferous *T. lineatum* and *T. rufitarsus* is partially explained by such a phenomenon. The latter two species would avoid trembling aspen trees infested by *T. retusum* because of the presence of salicylaldehyde emitted in combination with lineatin (Figure 2.10 and 2.14). In turn, *T. retusum* would avoid conifers attacked by either *T. lineatum* or *T. rufitarsus* or both together because of the presence of *alpha*-pinene (and probably other conifer monoterpenes) emitted with lineatin (Figure 2.12). A similar differential response to *alpha*-pinene was found in Europe, where it was attractive to *T. lineatum* (Bauer and Vité, 1975), but repellent to the angiosperm-infesting *T. domesticum* and *T. signatum* (Klimetzek et al., 1980; Nijholt and Schönherr, 1976).

I was not able to answer the question of how semiochemical-based reproductive isolation could occur between *T. lineatum* and *T. rufitarsus*. Reciprocal GC-EAD analysis of heterospecific volatiles failed to reveal any species-specific chemical that distinguishes these sometimes syntopic species. Because GC-EAD analysis with ambrosia beetles is to a degree an uncertain art, some compounds may possibly not have been identified, because they were not present in sufficient quantities to excite the antennae tested. I found galleries of both species less then 10 cm apart in the same standing lodgepole pine trees attacked by mountain pine beetle, suggesting no reciprocally-repellent volatile synomones. Although such compounds may yet be discovered, it is also possible that close range separation is achieved by some other mechanism, e.g. stridulation (Rudinsky and Michael, 1973; Ryker and Rudinsky, 1976), or non-volatile gustatory chemicals. In fact pheromone specificity might be reduced in species that use other signals or cues. With identical pheromones, *T. lineatum* and *T. rufitarsus* may also maintain reproductive

isolation through temporal separation of flight and attack periods. *Trypodendron rufitarsus* initiates flight very early in the spring at temperatures as low as 8°C (Ian Wilson, Parks Department, Kelowna, B. C., pers. comm., pers. obs.), while *T. lineatum* begins to fly considerably later at a threshold temperature of 15.5 °C (Chapman and Kinghorn, 1958; Daterman et al., 1965). It is also conceivable that the two species release the pheromone at different times of the day as for example the artichoke plume moths, *Platyptilia carduidactyla* (Riley) and *P. williamsii* (Grinnell), which use an identical sex pheromone but release it at different times of the night (Haynes and Birch, 1986). Because the habitat of ambrosia beetles is three-dimensional, one would expect less selection pressure to develop reciprocally-repellent synomones than in bark beetles that must compete for a more limited two-dimensional habitat (Borden et al., 1981). Alternatively, mechanistic isolating mechanisms, e.g. incompartibity of copulatory organs can prevent interbreeding of species. In fact the male genital capsules are species specific and used as one of the key taxonomic characters to distinguish among species in the tribe (Wood 1982).

It is unclear why none of the other five host volatiles tested other than salicylaldehyde had any effect on *T. retusum* (Figure 2.13), nor is it clear how *T. retusum* avoids non-hosts such as paper birch. Benzyl alcohol, 1-hexanol, benzaldehyde and nonanal were repellent to the mountain pine beetle when released from attractant-baited traps (Borden et al., 1998). However in Experiment 9, *T. lineatum* was most attracted to baits that contained benzaldehyde and nonanal in addition to lineatin and saliclyaldehyde. This is a surprising result, because not only is it contradictory to results with coastal *T. lineatum* (Borden et al., 2001), but also *T. lineatum* was attracted to the traps despite the

presence of salicylaldehyde, which had a strong repellent effect in Experiments 5 and 6 (Figure 2.10).

Host selection, non-host avoidance, mate finding and avoidance of heterospecific insects is a series of complex interactions of environment, physiology, and behaviour in which a vast quantity of chemical information must be processed, including detection of host kairomones (Byers, 1995; Miller and Borden, 1990b), non-host volatiles (Byers et al., 1998; Deglow and Borden, 1998a; Deglow and Borden, 1998b; Huber and Borden, 2001; Poland et al., 1998), sex and aggregation pheromones (Borden, 1985), and reciprocally-repellent synomones (Borden, 1997; Evenden et al., 1999). My results demonstrate that specificity in pheromone chemistry, avoidance of non-host volatiles and enhanced synergistic interactions between pheromones and host volatiles can explain in part the behavioural basis of reproductive isolation among four native sympatric ambrosia beetles in B.C. The lack of complete semiochemical-based segregation suggests that *Trypodendron* speciation occurred only recently, with selection pressure favouring the integration of pheromonal and kairomonal signaling as separating mechanisms over the evolution of new pheromones. Alternatively if hybridization among sympatric *Trypodendron* species is not possible, no selection pressure for divergence of chemical mating signals is necessary (Butlin, 1995).

3 Fungi Associated with *Trypodendron* spp.

3.1 Introduction

The ability to grow food is not unique to humans but has independently evolved in three insects groups: ants, termites and ambrosia beetles. Fungus-farming is found in ca. 200 species of nearctic attine ants, 330 species of Palaearctic termites, and 3,400 species of ambrosia beetles worldwide (Mueller and Gerardo, 2002). The ability to farm their own food in a mutualistic and apparently obligatory relationship with specific symbiotic fungi may explain in part why these insects have become major agricultural, household, and forestry pests (Batra and Batra, 1979; Beaver, 1989; Farrell et al., 2001; Mueller and Gerardo, 2002; Mueller et al., 1998; Wilson, 1971).

In ambrosia beetles (Coleoptera: Scolytidae and Platypodidae), fungal symbionts are vital, because they provide the nutritious "ambrosia" that is essential for larval development and completion of the beetles' life cycle (Batra, 1972; Beaver, 1989; French and Roeper, 1972). Larvae of all instars feed exclusively on ambrosia fungi that the adults inoculate into new breeding galleries. These fungi cause substantial economic damage. Depending on the habits of their beetle vectors, they may weaken or kill trees (Appendix I), and degrade wood products due to stain produced by enzymatic action of the fungi. Degradation caused by the darkly-stained galleries and the surrounding wood exceeds $ 100 million per year on the British Columbia coast (McLean, 1985), and may approach $ 200 million (Lindgren and Fraser, 1994b).

For ambrosia fungi the insect vector offers a means of reliable transport to and occupation of suitable and often ephemeral habitats. The fungal spores are protected from desiccation during flight and hibernation inside the beetles' fungal storage structures, or

mycangia (Batra, 1963; Batra, 1967). After inoculation into the wood of a new host, the fungus also benefits from the beetles' tunneling, which aids rapid spread of the mycelium (Batra, 1972; Beaver, 1989). Browsing by the beetles stimulates the growth of the ambrosia fungi, which by antagonism inhibit the growth of foreign fungi (Francke-Grosmann, 1967). In turn, ambrosia fungi provide food for the beetles, breaking down lignin and cellulose into simpler organic molecules which can be easily assimilated (Baker, 1963; Beaver, 1989; Haack and Slansky, 1987). One limiting factor for reproductive success and larval development is the amount of dietary nitrogen and phosphorous (Ayres et al., 2000). The fungi ensure a nitrogen rich diet for the beetles by sequestering nitrogen from the wood and recycling nitrogen excreted by the beetles (Ayres et al., 2000; Batra, 1963; Batra and Downing Michie, 1963), providing a higher quality source of protein than the wood itself. In addition the fungi provide B-vitamins and sterols that are necessary for pupation but cannot be synthesized by the beetles (Baker, 1963; Haack and Slansky, 1987; Kok et al., 1970; Norris, 1979). In the absence of mycangial fungi, the southern pine beetle *D. frontalis* laid only half the normal average number of eggs (Goldhammer et al., 1990), and it might be impossible for ambrosia beetles to produce any progeny at all. The fungi can also assist the beetles in the invasion of living trees or tissue by reducing the host's defense mechanism in the vicinity of fungal growth (Beaver, 1989; Paine et al., 1993). The moisture content of the phloem and wood can also be altered by fungi associated with bark beetles and thereby assist beetle colonization, creating a favorable microclimate for development of the insect (Francke-Grosmann, 1967; Webb and Franklin, 1978; Whitney, 1982). The fungi may also assist in converting host chemicals to pheromones by oxidation and detoxifying host chemicals as

shown for some bark beetles (Brand et al., 1976; Leufven, 1991). Insect-plant interactions that involve microbial mutualists, such as plant pathogenic fungi (Paine et al., 1997), can expand the capacity of insects to use plant resources (Hölldobler and Wilson, 1990; Mueller et al., 1998) and thus may enhance the rate of species diversification (Farrell, 1998). This may occur in part through widening the beetles' host range, because fungi can create favourable conditions in a wide range of host trees, on which the beetles are no longer directly nutritionally dependent (Beaver, 1989). Conversely, if the fungi are substrate-specific the beetles' host range may be limited.

While enlarging the larval chambers first and second instar ambrosia beetle larvae swallow some of the excavated wood and pass it through the gut (Baker, 1963). It is not clear if the ingested wood is digested and therefore has nutritional value or whether this is simply a way to manage the excavated wood particles. During gallery elongation, adults also ingest wood, but females must feed on fungi prior to ovary maturation (Beaver, 1989; Kingsolver and Norris, 1977). Adults may also feed on the fungi at other times, possibly as a farming practice and not for nourishment. Caring for the young may involve active placement of a fungal mass in front of the larval cradle opening (Hubbard, 1897). The presence of an active female is essential for healthy brood development. If the female beetle dies, and in some species either parent, the brood larvae die too, because they are apparently unable to control the fungal growth within the gallery (Beaver, 1989; Norris, 1979).

Usually only one generation of beetles breeds in a host, because the habitat is too altered or degraded to support additional generations. Therefore, each generation must disperse to find a new host, which has implications for the association between particular

fungi and their insect vectors (Beaver, 1989). Mature adults actively transport the fungal spores either by ingestion before dispersal or more commonly by gathering spores into their mycangia, glandular cuticular invaginations in the integument (Batra, 1963; Francke-Grosmann, 1956), where they are stored during dispersal and overwintering diapause. It appears that only spores of certain species survive in the mycangia, which, in a poorly understood process, selectively eliminate "unwanted fungi" (Barras and Perry, 1971; Francke-Grosmann, 1967; Paine and Birch, 1983).

Mycangia are often lined with at least two different types of secretory glands, the palisade glandular cells and the ductile-associated glandular cells (Barras and Perry, 1972; Happ et al., 1971), which produce waxy substances, fatty acids, phospholipids, sterols and amino acids, that provide moisture and nutrition for the stored fungus within the mycangial lumen. The activity of these glands varies with seasonal changes and the activity of the beetle (Schneider and Rudinsky, 1969), and they are most active during the flight of the beetles (Beaver, 1989) and during active boring (Baker, 1963). In addition to controling the growth of the essential mycangial fungi and suppressing other fungi, they may also stimulate transformation of the mutualistic fungi to the asexual form (Norris, 1979).

The growth and multiplication of fungus inside a mycangium is positively correlated with the development and secretory activity of the glandular cells (Schneider and Rudinsky, 1969). Thus the mycangia serve not merely for transport, but also provide a culture medium for the fungus (Barras and Perry, 1971; Francke-Grosmann, 1967; Harrington, 1993).

The type of mycangium is often specific to a particular genus (Beaver, 1989;

Dowding, 1969; Francke-Grosmann, 1967; Whitney, 1982). In all adult female *Trypodendron* spp., the mycangia are glandular ectodermic tube-shaped invaginations extending into the prothorax as a pair of u-shaped tubes opening above the base of the prothoracic coxae between the integument and the muscle. The tubes are finely sculptured, with strongly sclerotized spines (Abrahamson et al., 1967; Abrahamson and Norris, 1966; Francke-Grosmann, 1958; Francke-Grosmann, 1967). Adult females deposit spores on the exposed sapwood while excavating new breeding galleries. The adult beetles actively crop and feed on the fungal cultures and may have other mechanical and chemical mechanisms to keep the cultures pure (French and Roeper, 1972). For example, beetles and fungi produce antibiotics to ensure the growth of the right composition of microorganisms (Nakashima and Iizuka, 1982; Whitney, 1982). The entry of spores into the mycangia before dispersal to overwintering sites may to be passive, although Batra and Batra (1967) suggested that rocking movements of adults in the parental gallery forces spores into the mycangia (Batra and Batra, 1967). The expelling of spores seems to be driven by movements of the thoracic muscles when the female starts a gallery in a new host (Schneider and Rudinsky, 1969).

The fungal growth in the galleries is mycelial and the hyphae may penetrate the xylem and phloem, but sporulation probably only occurs in the gallery and may be stimulated as a result of the beetles, browsing activity (Batra, 1966). Around the tunnel walls the fungal hyphae form a stroma from which a palisade of spore-bearing hyphae projects into the lumen of the tunnel. These hyphae are browsed by both adults in the gallery and larvae inside their niches (Baker, 1963; Batra, 1967). The shiny spores (originally thought to be yeast) are often arranged in tangled chains of moniliform cells

on the gallery walls (Baker, 1963). This dense hyphal layer and the fungal mass on which the larvae feed are referred to as 'ambrosia', as are the monilioid yeast-like vegetative hyphal fragments, found in the mycangia and the galleries (Batra et al., 1986). Ambrosia fungi have a small range of optimal growth temperatures (Nakashima et al., 1987) and seem to develop as a result of mechanical and chemical manipulation which may involve secretions of the beetles and their larvae (French and Roeper, 1972). In support of mechanical stimulation, artificial cutting of hyphal tips of the mycelial form of *Ambrosiella hartigii* Batra produced a change from mycelia to the ambrosial form (Batra, 1967). In agar culture the monilioid ambrosia growth is often lost, the hyphae appear cottony, and the medium is stained dark either by dark hyphae or by oily exudates (Baker, 1963). The ambrosia fungi are pleomorphic and can easily change their morphs when the medium changes or as a result of mechanical manipulation (Batra and Downing Michie, 1963; Funk, 1965). Ambrosia cells multiply extensively in the tunnel system while the parents are present, but often fail to germinate when plated on artificial media (Batra, 1963). The dominant fungal species may differ according to location within the gallery system and the duration of occupation by the beetles (Batra et al., 1986; Beaver, 1989). After the brood adults leave, the gallery system is rapidly overgrown with fungi that were suppressed and apparently not used by the resident beetles. All of these features can lead to misidentification based on morphological structures, and misinterpretation of which species are the true and essential ambrosia fungi. Isolations from the mycangia directly and combining molecular techniques with traditional taxonomy should allow for unambiguous identification.

Fungi in more than 30 genera have been isolated from ambrosia beetles (Baker,

1963; Batra, 1963; Francke-Grosmann, 1967). Many ambrosia genera belong to the ascomycete group known as the ophiostomatoids (Ascomycetes: Ophiostomataceae) including the genera *Ophiostoma*, *Ceratocystis* and their associated anamorphs (Beaver, 1989; Farrell et al., 2001; Malloch and Blackwell, 1993; Wingfield et al., 1993). For the genus *Trypodendron* several attempts have been made to establish the range of symbiotic fungi (Table 3.1). One problem with previous studies is that the spores were often isolated from the beetles' galleries or body surface, or from crushes of whole bodies or the digestive system (Bakshi, 1950; Batra, 1963, 1967; Francke-Grosmann, 1956; Hinds and Davidson, 1972), but not from mycangia, which are most likely to yield only one or two specific fungi associated with particular beetle species (Six and Paine, 1999). Another problem is that most isolations were done over 30 years ago, when a true ambrosia fungus was considered to produce "in culture the same ambrosial stages as in the tunnel". It is now known that the pleomorphic ability of the fungi renders this criterion invalid (Francke-Grosmann, 1967). Overlooking seasonal morphological changes and altered fungal species composition (Bakshi, 1950; Batra, 1963; Funk, 1965; Hinds and Davidson, 1972) also leads to misinterpretation.

The genus *Trypodendron* in B.C. includes four native and one introduced species that are mutually sympatric. Two species, *T. lineatum* and *T. rufitarsus*, infest a variety of conifer hosts in their range, while *T. betulae* and *T. retusum* inhabit the angiosperm genera *Betula* (Betulaceae) (birch) and *Populus* (Saliaceae) (poplar and aspen), respectively.

The heartwood of conifers is composed of dead cells, containing large amounts of phenolics, and apparently is not a suitable substrate for ambrosia fungi. Thus the habitat

TABLE 3.1 Species of fungi previously associated with *Trypodendron* species.

Beetle species	Fungal species *	Notes on isolation	Reference(s)
T. retusum	*Ceratocystis retusi* *C. brevicollis* *Ambrosiella ferruginea* *C. leucocarpa* *C. tremoloaurea* *C. piceae* *Graphium* ssp. *C. crassivaginata* *Cytospora chrysosperma*	Isolated from adults, pupae, cradles and galleries, but not mycangia	Hinds and Davidson (1972)
T. rufitarsus	*A. ferruginea*	No information	French and Roeper (1972)
T. lineatum	*Monilia ferruginea* *Ceratocystis bicolor*	Isolated from galleries and mycangia	Batra and Downing-Michie (1963); Funk (1965)
	C. piceae *Leptographium lundbergii* *Oedocephalum lineatum*	Isolated from adults and galleries, but not mycangia	Bakshi (1950)
	M. ferruginea	No information	Mathiesen-Käärik (1953)
T. domesticum	*Ceratocystis ambrosia* *M. ferruginea* *Endomycopis fasciculate* *Trichosporium tingens* *C. bicolor*	Isolated from adults and galleries, but not mycangia	Bakshi (1950)
T. lineatum *T .domesticum* *T. retusum* *T. betulae* *T. signatum*	*A. ferruginea*	Isolated from tunnels and/or mycangia	Batra (1967)

* Current placements of taxa and authors of latine names of fungus species:
Ceratocystis retusi, Ceratocystiopsis retusi (R. W. Davidson & T. E. Hinds) H. P. Upadhyay; *Ceratocystis brevicollis, Ophiostoma brevicolle* (R.W. Davidson) de Hoog & R. J. Scheff; *Ambrosiella ferruginea* (Mathiesen-Käärik) Batra; *Ceratocystis leucocarpa* R. W. Davidson; *Ceratocystis tremoloaurea, O. tremoloaureum* (R. W. Davidson & T. E. Hinds) de Hoog & R. J. Scheff; *Ceratocystis* piceae (Münch) B.K. Bakshi, *Ophiostoma piceae* (Münch) Syd. & P. Syd.; *Ceratocystis crassivaginata, Ceratocystiopsis crassivaginata* (H.D. Griffin) H. P. Upadhyay; *Cytospora chrysosperma, Valsa sordida* Nitschke; *Monilia ferruginea, Ambrosiella ferrugine* (Mathiesen-Käärik) L.A. Batra; *Ceratocystis bicolor, Ophiostoma bicolor* R.W. Davidson & D.E. Wells; *Ceratocystis ambrosia* B.K. Bakshi; *Ceratocystis pilifera* (Fr.) C. Moreau; *Endomycopis fasciculate, Pichia fasciculata* (L.A. Batra) Abadie & Lehodey; *Trichosporium tingens, Ambrosiella tingens* (Lagerberg & Melin) L. A. Batra, *Leptographium lundbergii* Lagerb. & Melin; *Oedocephalum lineatum* B.K. Bakshi; *Trichosporium tingens* Lagerberg & Melin.

in healthy conifers the sapwood may contain toxic constitutive resin in resin canals that may be severed by attacking beetles, or parenchyma cells in the sapwood may produce an even more toxic traumatic resin in response to insect or fungal invasion (Coyne and Lott, 1976; Delorme and Lieutier, 1990; Paine et al., 1997; Raffa, 1991). Therefore conifer-infesting ambrosia beetles avoid host resistance by infesting the sapwood of very weak hosts, i.e. logs, stumps and dying trees.

Unlike the sapwood of conifers, the sapwood of angiosperms lacks extensive oleoresin-based constitutive and induced resistance mechanisms, although other resistance mechanisms, e.g. the production of phenolics, may exist. In addition, the heartwood of many angiosperm trees is indistinct, and may not be nearly as toxic a habitat as conifer heartwood. Thus, because angiosperm-infesting ambrosia beetles are not nutritionally dependent on living phloem or sapwood, they can bypass potential host resistance by penetrating deep into the dead heartwood of a living, healthy tree.

North American *Trypodendron* spp. are usually described as attacking only 'unthrifty' host trees or logs (Wood, 1982b), but my recent observations in the southern interior of B.C. may change this perception (Appendix I). *Trypodendron retusum* not only occurs on wind-broken trembling aspen, *Populus tremuloides* Michx., and trees damaged by vertebrates or lightning, but also on apparently healthy, standing aspens of various sizes. In some cases *T. retusum* infestation apparently causes patch kills that result in spindle-shaped lesions of exposed dead wood. I have also found *T. betulae* in standing paper birch, *Betulae papyrifera* Marsham, with no visible signs of stress. The ability of the beetles and fungus to attack healthy angiosperms might be facilitated by the potential of the fungus to grow inside a tree to such a degree that it compromises the tree

growth and may even kill the tree. One goal of this study was to test the fungi isolated from *T. retusum* for their potential pathenogenicity to trembling aspen by inoculating them into fresh logs and standing trees. Because of chemical and physical differences between *Betula* and *Populus* spp. it is possible that the ambrosia beetles inhabiting these hosts harbour species-specific fungi that contribute to the reproductive isolation of their beetle symbionts. In no case have the fungi associated with any of B.C.'s *Trypodendron* spp. been investigated using currently accepted methodology.

Attack by ambrosia beetles is almost invariably associated with discolouration of the invaded tissue which probably results from a response by the host tree to symbiotic microorganisms (Faulds, 1973). This "stain" is in addition to the dark coloration of the fungal mycelium itself, and implies that at least some degree of pathogenicity is needed to overcome even weak hosts. Although some ophiostomatoid fungi, e.g. *O. ulmi* (Buisman) Nannf., *O. novo-ulmi* (Brasier) are important and destructive pathogens, most species of *Ophiostoma* are weak parasites or saprophytes. Even weakly pathogenic species, such as *O. ips*, current name *Ceratocystis ips* (Rumbold) C. Mureau, *O. minus*, current name *Ceratocystis minor* (Hedgcock) J. Hunt, *O. penicillatum*, (Grosmann) Siemaszko, and *O. piceae* (Münch) H. and P. Sydow, impart serious economic losses by causing blue stain in harvested timber (Seifert, 1993), especially associated with bark beetles (Gibbs, 1993).

The increasing frequency of observations of ambrosia beetle attack on living trees (Appendix 1) suggests that the pathenogenicity of ambrosia fungi warrants more careful examination than in the past (Bhagwandin, 1993; Browne, 1965; Costilla and Venditti, 1992; Finnegan, 1972; Kamata et al., 2002; Kent and Simpson, 1992; Kovach and

Gorsuch, 1985; Nord and McManus, 1972; Wood, 1982b). For example, mass attack by the exotic ambrosia beetle, *Xylosandrus crassiusculus* (Motschulsky), can kill North American ornamentals and fruit trees, but pathogenicity of its fungal partners has not been investigated (Chapin and Oliver, 1986; Deyrup and Atkinson, 1987; Kovach and Gorsuch, 1985; Wood, 1982).

Fungi associated with bark and ambrosia beetles may kill a tree or a portion of it by producing toxins or occluding the xylem vessels or tracheids with mycelium, spores, or polysaccharides. Furthermore, tyloses may be formed by cells exposed to fungal enzymes. In living trees this symptomatic condition causes vascular wilt (Agrios, 1988). A mechanical girdling effect by ambrosia beetles only occurs with mass attack. Successful colonization of living wood depends largely on its moisture content. If the wood is saturated with water, oxygen becomes the limiting factor, while water shortage may limit solubility and metabolic activity of hydrolyzing enzymes and diffusion of metabolites (Zabel and Morrell, 1992). For example *O. minus* grows very slowly in fresh logs but loss of 10% of the water content allows rapid penetration of the fungus (Gibbs, 1993). Loss of water by the host may be necessary in general before invasion by ophiostomatoid fungi can occur (Gibbs, 1993). In addition, altered moisture content may alter competitive interactions between beetle fungal symbionts (Klepzig et al., 2004). In turn the beetles' larval development is enhanced by fungal colonization which reduces inner bark water content (Graham, 1967). Commonly, assumptions about fungal growth in living host tissue and host- fungus interactions are based on *in vitro* experiments, with the fungi growing on various nutrient rich agar media, on pieces of wood maintained on agar at high humidity, or on natural substrates that are altered by heat, autoclaving,

chemical treatment, or irradiation (Gibbs, 1993; Uzunovic, 1996; Uzunovic and Webber, 1998). Because I observed *T. retusum* commonly attacking living trees, I hypothesised that one or more species of its symbiotic fungi was at least moderately pathogenic. Therefore, one of my objectives was to test this hypothesis by investigating the growth of its fungal associates in freshly cut trembling aspen billets that were unaltered by drying or sterilization. This was to provide insight into the ability of these fungi to invade and colonize living host tissue. Another objective was to explore whether the fungi associated with *T. retusum* have the pathogenic potential to kill healthy trees or to compromise their growth.

Growth *in vitro* may sometimes reflect the pathenogenicity or ability of fungal species to colonize *in vivo*, but the logical continuation of laboratory studies is inoculation of fungi into living tissue. Inoculating fungi into billets freshly cut from living trees allows investigation of the ability of a fungus to colonize living tissue capable of defence, while maintaining controlled conditions in a growth chamber. Using this technique, (Uzunovic and Webber, 1998) found that *Ceratocystis coerulescens* (Münch) Bakshi grew almost three times faster longitudinally in woody tissue then on nutrient agar at the same temperature, contradicting common assumptions and indicting that this blue stain fungus is stimulated by growing on a natural substrate.

3.2 Objectives

The four objectives in this chapter were:

1) to develop a reliable method for isolation of ambrosia fungi directly from the mycangia of *Trypodendron* spp.;
2) to isolate and to differentiate the fungi that are associated with the four native and

one introduced *Trypodendron* spp. found in B.C., based on basic morphological descriptions and on DNA sequence data;

3) to investigate the ability of fungi associated with *T. retusum* to invade colonize host tissue, using freshly cut billets from trembling aspen trees; and
4) to explore whether the fungi associated with *T. retusum* have the potential to kill healthy aspens or to compromise their growth.

3.3 Methods

3.3.1 General Culturing Methods

Culture media were routinely autoclaved for 20 min at 121°C and 1034.1 KN m^{-2} (15 psi). After autoclaving the bottles with media were left to cool to about 50°C in a laminar flow hood, and the media were poured aseptically into sterile 90 mm Petri dishes, approximately 20 mL of agar per dish. The Petri dishes were left over night in the laminar flow hood and stored before use for four weeks under polyethylene plastic bag covers at room temperature.

Two growth media were used:

2% Oxoid Malt Extract Agar (MEA):

33 g MEA (CM 59)

10 g Agar, technical agar No 3 (L13)

1000 L distilled water

Oatmeal Agar:

30 g "Robin Hood" large flake oats

20 g Agar, technical agar No 3 (L13)

800 L distilled water

For oatmeal agar, 30 g of oats were added to 800 mL of boiling distilled water in a 1-2 L beaker or flask and simmered under constant stirring with a heavy stirrer for about 60 min. The mixture was filtered through muslin and the extract made up to 1 L of liquid by adding distilled water and divided into two 1 L flasks. Agar was added under stirring and then autoclaved and poured the same way as above.

A selective medium was also used:

Oxoid Malt Extract Agar plus Actidione and Streptomycin (A+S):

33 g MEA (CM 59)

10 g Agar, technical agar No 3 (L13)

1000 L distilled water

50 ml Cycloheximide (=Actidione) solution

25 ml Streptomycin solution

Malt extract and technical agar were acquired from Oxoid Ltd, Basingstoke Hampshire, England, streptomycin sulphate from Sigma, S-6501 Lot 22H0448, and cyoheximide from Sigma, C-7698 Lot 97H0474. Cycloheximide inhibits the growth of many fungi but not fungi in the genera *Ophiostoma* and their associted anamorphs *Leptographium*, *Pesotum*, and *Sporothrix* (Harrington, 1981). Cyloheximide solution is prepared by diluting 1 g of Cycloheximide in 500 mL of water, and fifty mL of this solution is added into the agar before autoclaving. Streptomycin solution is prepared by diluting 1 g of Streptomycin sulfate in 100 mL of sterile water and filtered into cooled autoclaved agar just before pouring. Streptomycin inhibits the growth of bacteria. For all experiments, each primary isolate was first grown on plates with MEA and plates with A+S. The subcultures were plated on MEA by centrally placing an approximately 2 x 2

mm inoculum taken from the edge of a 3-10 day old culture. Cultures were allowed to grow at room temperature for 3-21 days, depending on how fast they increased in size.

Petri dishes containing cultures were stored in the dark at 3-7°C wrapped in polyethylene bags to maintain cultures for short periods. Isolates were stored for longer under sterile water (1-3 years) or under mineral oil (more than 2 years) in sterile cryovials at 3-4°C. Representative isolates used in this study are maintained in the culture collection at Forintek Canada Corp. Western Laboratory, 2665 East Mall, Vancouver, B.C., V6T 1W5 and at the Department of Wood Science, Faculty of Forestry, University of British Columbia, Vancouver, B.C., V6T 1Z4.

3.3.2 Isolations from Beetles and Galleries

Live beetles were collected early in the spring at the time of the dispersal flight either from clean lineatin-baited traps upon landing or excised from host logs or trees shortly after attack. During the breeding phase, when excavation of tunnels and egg niches was under way, adults were excised from their hosts. Larvae and pupae were taken from excavated gallery systems as long as eggs were still being laid. For every beetle species two adult females and males, pupae and larvae, were individually vortexed for 1 min in 1 mL sterile water and dilution series of 1:5, 1:50 and 1:500 were made. A 100 µL aliquot from each dilution was spread onto one MEA and one A+S plate with a sterile glass rod, incubated in darkness at 20°C and assessed after 1-3 days. The numbers of fungal and yeast/bacterial colonies were counted to give an estimate of the number of spores carried by each insect. A selection of emerging single spore colonies were transfered onto separate MEA plates for further studies. Sampling from the galleries was done by picking up hyphae of superficially growing mycelia or spores with a sterile

needle and placing them onto agar plates. Any growth that appeared after 2-10 days was subcultured onto MEA.

All subcultured isolates from all sources were characterized and grouped based on their colour and colony growth characteristics, e.g. colony margins, pattern and rate of growth on plates, as well as the size, shape and colour of teleomorph and anamorph if present. Photographs were taken of representative colonies and structures. This work was done in collaboration with Dr. Adnan Uzunovic, Forintek Canada Corp. Western Laboratory, 2665 East Mall, Vancouver, B.C., V6T 1W5, an experienced mycologist with extensive experinence with this group of fungi. Representative isolates were used for DNA sequencing and phylogenetic determination. Sequencing analyses were performed by Dr. Jae-Jin Kim, Department of Wood Science, Faculty of Forestry, University of British Columbia, Vancouver, B.C., V6T 1Z4.

3.3.3 Isolations from Mycangia

The aim of this endeavor was to excise single mycangia and isolate the fungi contained in them. All instruments used were flame-sterilized and kept in 70% ethanol until use. Dissections were performed in sterilized glass Petri dishes lined with sterile filter paper. Each live female was first immersed in 70% ethanol for 10-20 sec. The legs were pulled out of the prothorax which was then separated from the head and the abdomen and bisected with a scalpel. Each side was treated separately to isolate a single mycangium, and all cuticle and tissue was carefully removed from the mycangial tube. A new sterile instrument was used for each manipulation. A drop of ethanol remaining on an instrument was allowed to spread over the dissection, maintaining a sterile work field. The isolated mycangial tube was carefully squeezed with forceps to expel the fungal

mass, which was then transferred into a sterile plastic test tube and vortexed in 1 mL of sterile water. One μL aliquots of 1:5 and 1:50 dilutions of fungal suspensions were spread on one plate each of MEA and MEA+A+S media. Plates were left at room temperature and artificial light during the day and in the dark at night. Any growth, which appeared, after 2-10 days was subcultured onto MEA plates.

3.3.4 Source of Beetles and Galleries

In 2000, *T. retusum* were excised from a standing attacked trembling aspen tree collected north of Brookmere, B.C. (49°52'N, 120°55' W), on 26 June. Eggs were still being laid, and larvae and pupae were present in the galleries. *Trypodendron betulae* were excised from a fallen paper birch log near Salmon Arm, B.C. (50°47'N, 119°12' W) on 01 July. Adults, eggs, larvae and pupae were present. *Trypodendron lineatum* were collected in individual sterile vials upon landing on lineatin-baited multiple-funnel traps, south of Brookmere (49°48'N, 120°51' W), B.C. on June 26 and July 21. In 2001: *T. rufitarsus* and *T. lineatum* were excised from a bolt cut on 12 June 2001 near Princeton, B.C. from a lodgepole pine tree that was attacked by the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, in 2000, and baited with lineatin on 18 January 2001. *Trypodendron domesticum* were collected in individual sterile vials upon landing on multiple-funnel traps baited with *ESR*- and *ZRR*-tirathol at Simon Fraser University (49°17'N, 122°55' W), in March. *Trypodendron domesticum* and *T. betulae* were excised from a bolt cut on 20 June from a standing paper birch log in Richmond Nature Park, BC (49°9 'N, 123°0' W). Adults, eggs, larvae and pupae were present.

When fungi were transferred from beetle-infested host material directly, samples were taken from the fungal mass lining the beetles' tunnel walls and from the adjacent

stained wood of freshly exposed gallery systems. Also split wood pieces containing gallery walls were incubated at room temperature in humid chambers for up to one week and visible fungal growth was subcultured on MEA.

3.3.5 Growth Rate Procedures

Inoculum plugs approximately 2 x 2 mm were taken from the edge of growing colonies. They were placed in the center of Petri dishes containing 20 mL of MEA dispensed 1-2 days earlier to let the surface dry and to check against contamination. Inoculated plates were incubated in the dark at 20-21°C. The first growth measurement was taken 2-3 days later along two perpendicular diameter lines marked with a diamond pen on the Petri dish base. A second measurement was made after 2-3 additional days along the same diameters. Growth rates were calculated as the mean radial increment per day with two replicates per isolate. Photographs were taken after 10 and 25 days of growth.

3.3.6 Mating Procedures

A mating test was used to test if isolated fungi were *Ophiostoma piceae* (Münch) H. and P. Sydow. *Ophiostoma piceae* is heterothallic and opposite mating types must be brought together to produce the sexual fruiting bodies referred to as perithecia. For this test, two confirmed Canadian opposite mating types (OpA AU 122-5 and OpB AU135-4) (Uzunovic et al., 2000) were mated on sterile lodgepole pine sapwood wafers against representative isolates from *T. retusum*, *T. betulae* and *T. lineatum*. The plates with wood wafers were incubated at 20°C in darkness until the colonies met, then placed at room temperature and in diffuse light for a further 2-4 weeks. Drops of sterilized water were

added when needed to replace moisture in wood pieces and to keep a humid environment in the Petri dishes. To confirm mating, the meeting lines between colonies were checked after approximately 4 and 6 weeks for perithecia using a dissecting microscope with substage illumination.

Pairs of randomly chosen isolates from individual beetles were also mated as above against the remaining isolate from the same beetle to check for perithecial production, significant taxonomic features in identifying *Ophiostoma* species. In addition, two isolates were tested for self mating to determine if the fungal species were homo- or heterothallic. These mating tests were performed on an approximately 2 x 3 cm pieces of sterile wood from the host tree species, which were placed on MEA and on oatmeal agar. Prior to the test the wood pieces were submerged in 70 % ethanol for 2 min then air dried under a laminar flow hood and surface flamed.

3.3.7 Morphological Studies, DNA Sequencing and Phylogenetic Analysis

The morphological identifications and DNA analysis from cultures originated from single spore isolations was performed by Dr. Jae-Jin Kim, Department of Wood Science, Faculty of Forestry, University of British Columbia, Vancouver, B.C., V6T 1Z4. For reference purposes, type strains were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, and the culture collection of University of British Columbia, Vancouver, B. C. Morphological features were observed on fungal structures produced on 2 % MEA (20 g Difco malt extract, 15 g Difco agar, and 1000 mL distilled water) and on sterile lodgepole pine sapwood wafers. Isolates for identification were organized into groups based on described cultural characteristics (Harrington et al., 2001; Jacobs and Wingfield, 2001; Upadhyay, 1981). For light

microscopy, fungal structures were mounted in water and observed using a Zeiss Axioplan light microscope. Two or three representatives of each group were purified using single spore isolation methods (Uzunovic et al., 2000).

For genomic DNA preparations, fungal isolates were inoculated on 2 % OMEA media (33 g Oxoid malt extract agar, 10 g Oxoid technical agar, and 1000 mL distilled water) overlaid with sterile cellophane sheets (Bio-Rad) and grown for 7 days at room temperature. DNA extraction was carried out using the method described by Kim *et al.* (1999).

The ITS region and partial LSU rDNA were amplified using the primers ITS5/ITS4 (White et al., 1990) and LROR/LR3 (Vilgalys and Hester, 1990). The β-tubulin gene was amplified using the primer T10 and BT12 (Kim et al., 2003). PCR amplification was carried out with a total volume of 25 μL in 0.6 mL reaction tubes and a Touchdown Thermocycler (Hybaid). The reaction mix contained 1x reaction buffer (10 mM Tris-Cl [pH 8.0], 1.5 mM $MgCl_2$, 50 mM KCl), 80 μM of each deoxynucleotide, 20 pmol of each primer, 0.5U Taq DNA polymerase (Rose Scientific) and 100 ng genomic DNA. The standard reaction conditions were as follows: initial denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 52°C for 50 sec and DNA elongation at 72°C for 50 sec, and a final cycle of DNA elongation at 72°C for 10 min. PCR products were visualized by electrophoresis on a 1.4 % agarose gel containing ethidium bromide.

The PCR products were gel-purified using a Qiaquick Gel Extraction Kit (Qiagen). Gel purified PCR products were sequenced using the primers described above. Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer Inc.

USA) at the DNA Synthesis and Sequencing Facility, MACROGEN, Seoul, Korea.

3.3.8 Inoculum Preparation

Approximately 600 organically grown whole, large wheat kernels were soaked in tap water at 4°C overnight and then approximately 60 grains were put in 100 mL conical flasks. Surplus water was removed, and the flasks were plugged with cotton wool, covered with tinfoil and autoclaved for 20 min at 15 psi and cooled for 1 h. Five discs approximately. 6 mm in diam. of inoculum taken from actively growing on MEA were inserted into each flask and mixed with the grain. The flasks were incubated at 20°C in the dark for 20 days until the grains were thoroughly colonized. Drops of sterile water were added to the flask when needed to maintain a moist environment. Sample kernels were placed on MEA before and after the inoculation experiment to verify that the fungus was alive and uncontaminated. To check the consistency of inoculations two different isolates of the same species of each of the three test fungi were used: 1) SK 6 and SK 10, described as "brown with pesotum anamorph" with a typical *Ophiostoma* morphology (fungus A) and later (after the experiment was completed) identified as *O. piceae*, isolated from pupae and female adults, respectively; 2) SK 12 and SK 21 described as "large white fluff" (fungus B) and later identified as *O. piliferum* (Fries) Sydow & Sydow, both isolated from mycangia; and 3) SK 2 and SK 4 described as "small white fluff" (fungus C) and later identified as *O. quercus*, isolated from larvae and pupae, respectively.

3.3.9 Inoculation Procedure

Three trembling aspen trees were felled just east of Manning Park, B.C. (49°10'N, 120° 35' W), one on 14 February and two on 2 March 2001, wrapped in polyethylene bags and held outdoors at Forintek Canada Corp. Western Laboratory, 2665 East Mall, Vancouver, B.C., V6T 1W5. Each tree was cut into 6-7 straight billets at least 35 cm long and 15 cm diam. Immediately after cutting, the exposed ends of each billet were sealed with paint sealer (Instant Patch, Tremco Ltd, Canada) to prevent moisture loss and microbial contamination. Two discs approximately 2 cm thick were cut from each tree to measure the moisture content. Each billet was wrapped in a new polyethylene bag and held at 0°C until 6 March, when the billets were inoculated with fungi isolated from *T. retusum*. On each billet, fungi were inoculated alone or in combination, and into two types of holes, deep (3 cm) or shallow (the depth of the phloem-xylem interface) equally spaced around the log circumference 10-15 cm from the bottom end of each billet. Fungal isolates were inoculated as two pieces of pre-colonized wheat grain inoculum into holes drilled with a 5 mm diam. electrical drill bit sterilized in 70% ethanol and flamed before use. The bark surface around the holes was sterilized with an ethanol wipe and quickly flamed prior to inoculation. For single species inoculations, two kernels of the same isolate were used, and for combined inoculations, one kernel of each species was used. Each billet received four treatments out of seven possible (AA, BB, CC, AC, AB, BC, and OO for control) randomly assigned to the billets and to each treatment depth. Inoculated holes were covered with a piece of sterile gauze and with electrical tape placed around the billet over the holes protecting against desiccation. Control holes were treated the same as treatment holes except no inoculum was transferred. The logs were

incubated for 27 days in a growing chamber running at17°C and 80% RH.

3.3.10 Determination of Moisture Content

Moisture content was determined as a percentage of oven-dry weight (% odw), indicating the total amount of water present in the wood and as a percentage of saturated weight (% saturation), as given below.

$$\% \text{ odw} = \frac{\text{fresh weight} - \text{dry weight}}{\text{dry weight}} \times 100$$

$$\% \text{ saturation} = \frac{\text{fresh weight} - \text{dry weight}}{\text{saturated weight} - \text{dry weight}} \times 100$$

Comparison of the two values allows the amount of free water to be separated from the amount of bound water held in the wood in cell walls. The latter measure takes into account the density of the wood. Moisture content (mc) was estimated from two freshly cut discs, which were immediately wrapped in polyethylene bags and kept at 5°C until sampled. Each disc was split with a chisel and mallet giving eight small sapwood blocks of various sizes across the diameter of each. For determination of odw, each block was individually weighed for fresh weight, dried at 103°C for 24 h to constant weight, cooled for approximately 1 h in a desiccator over silica gel and then re-weighed. For determination of % saturation, freshly weighed blocks were boiled in water for 2 h, left in the water-filled container to cool overnight, during which they sank to bottom indicating full saturation, surface dried by blotting on filter paper, and re-weighed.

3.3.11 Sampling for Stain and Recovery of Inoculum

The billets were sampled destructively by transverse sectioning using a bandsaw into approximately 1 cm thick discs starting at the inoculation point and then at pre-marked intervals up the billet. The discs were used to estimate the extent of fungal growth by measuring the area of discolouration, and sampling to recover fungi from discoloured areas to estimate the ability of the different species of fungi to colonize the wood. Using Uzunovic and Webber's (1998) techniques, longitudinal growth was estimated as the distance from the inoculation point to the last disc with visible discolouration. The extent of the radial (depth) and tangential (lateral) growth from the point of inoculation was determined by the stain visible on the distal face of the first disc. Isolations to recover inoculated fungi were attempted from both visibly stained areas and areas adjacent to the stain. A sterilized scalpel was used to shave off the surface of the wood and 2-3 mm^3 wood chips were removed from the exposed wood and placed onto agar plates. After 2-3 days any colonies were subcultured onto MEA. Positions of all attempted isolations were recorded. Fungus A alone was sampled 15 times, B alone 24 times, C alone 30 times, AB combined 11 times, AC combined 14 times, BC combined 8 times and control drill holes 7 times. Five different billets and 25 discs were sampled.

3.3.12 Inoculation of Living Trees

Inoculations with fungi isolated from *T. retusum* were performed on 18-20 April, 2001, on apparently healthy intermediate-aged trembling aspen trees before bud flush 0.5 km east of Manning Park, B.C. (49°10'N, 122°55'W), along the first 500 m of East Gate Forest Road. The experimental trees were surrounded by a mixed stand of mature

lodgepole pine and trembling aspen trees. The trees were 10-15 m tall and had a mean diameter at breast height (dbh= 1.3 m) of 12.9 ± 1.5 cm.

Inoculum was prepared as in Section 3.3.8 and the same three fungal species from *T. retusum* were used, each represented by two different isoaltes. Sample wheat kernels were placed on MEA before and after inoculations to verify that the fungal cultures were alive and uncontaminated after being stored in sterile water for one year after isolation from the beetles. The night before the experiment wheat grains were put in sterile Eppendorf tubes in groups of 8-10 kernels for transportation to the field site.

Sixteen inoculation holes were drilled 3 cm deep at 45° with a 5 mm diam. electrical drill sterilized in 70% ethanol and flamed before use and between holes. A basal ring of four holes was made at approximately 1.3 m above ground, equally spaced around the tree circumference. The next three sets of four holes each were placed at 5 cm intervals up the bolt, alternately rotated by 45° or in line with the basal ring. The surface around the holes was sterilized with an ethanol wipe and fast flamed prior to inoculation. Fungal isolates were inoculated as three kernels of pre-colonized wheat grain inoculum. Fungi were inoculated alone (isolate A, B, or C) or all fungi in combination. Ten replicates of each treatment including an uninoculated control were prepared and assigned in a randomized block design to 50 trees spaced at least 5 m apart. Inoculated holes were protected against desiccation with a sterile cotton wad and sterile natural corks or sterile Q-tips with the ends cut close to the bark surface. Uninoculated control trees were otherwise identical to treatment trees.

Trees were observed throughout the following two years and all trees of one randomly chosen replicate were felled on 10 October, 2001 and bolts encompassing the

inoculation area were taken to Forintek Canada Corp. In the laboratory the bolts were examined for beetle attack, the presence of the corks or Q-tips and discolouration and shape of the bark indicating lesions. Three lesions per tree were sampled for fungal presence by using a sterile scalpel to excise 3 or 4 small wood chips from discoloured wood tissue around the drilled holes or from the adjacent unstained wood. The chips were plated individually on MEA and incubated at room temperature. Tissue was also sampled from discoloured areas inside the trunk and in a longitudinal direction away from the inoculation holes.

3.4 Results

3.4.1 Isolations

During the sampling process, approximately 200 isolates were subcultured and studied. In general, larvae and pupae bore more viable fungal spores than adults or mycangia. Mycangia yielded the fewest cultures, and sometimes none, even when the female was reproductively active. No isolates were obtained from the mycangia of females that had completed reproduction with only pupae or callow adults in the gallery, or from young adults ready to emerge from the natal gallery. If the mycangial content was deposited directly on media, no growth occurred. Also females captured in pheromone traps yielded fewer isolates than females excised from logs. If captured or excised adults were frozen for 2-14 days no isolations could be obtained. Most cultures had very little or no contamination, especially those generated from mycangia, which had no growth of bacteria or contaminating fungi and only occasionally moulds.

The white fungal layer lining the larval cradles (which become pupal cells) consistently yielded isolates that grew on media and were given the descriptive term

"white fluff" (Table 3.2, Figure 3.1), a term used also by Batra and Downing Michie (1963). The majority of isolates from mycangia had a similar "white fluffy" appearance. Another frequent type of isolate given the descriptive term "brown with pesotum anamoph" consistently produced pesotum anamorphs with a dark colouration of the agar plates appearing after 10 days, possibly the fungus responsible for the dark colouration of the gallery walls. *Trypodendron rufitarsus* and *T. lineatum* excised from same lodgepole pine tree, may have the fungi of the heterospecific beetle as contaminants, although there was no morphological evidence that would confirm this hypothesis. In contrast, wood from the tree that had been attacked the previous year by the mountain pine beetle and isolations from the adults of both species of ambrosia beetles revealed *Leptographium*-like anamorphs (Whitney, 1971) (not included in Table 3.2) typical of *Ophiostoma* associated with mountain pine beetles (Wingfield et al., 1993).

Pairing of two randomly chosen isolates from one beetle against the remaining others from the same beetle resulted in some successful matings, with perithecia being produced after 4-6 weeks. Isolates from *T. retusum* described as "brown with pesotum" (Table 3.2) were determined to be *O. piceae* because they produced perithecia with the known *O. piceae* testers. Nine of 11 isolates tested produced perithecia with either OpA or OpB type, and one produced very small and immature perithecia with both testers. No isolates from any other beetle species mated with these testers. Although later DNA analysis showed that the exotic *T. domesticum* is also associated with *O. piceae*, no mating test was performed with fungi isolated from *T. domesticum*. Most isolates were in the genus *Ophiostoma* (Wingfield et al., 1993) based on the following criteria: growth on cycloheximide, characteristic anamorphs (either pesotum or sporothrix ananmorphs), and

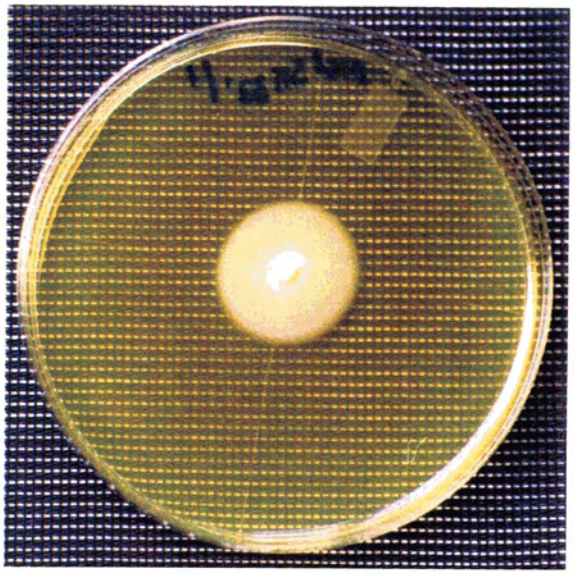
TABLE 3.2 Characterization of fungal isolates obtained from four native and one exotic *Trypodendron* species in BC. DNA analysis was performed by Dr. Jae-Jin Kim, Department of Wood Science, Faculty of Forestry, University of British Columbia, Vancouver, B.C., V6T 1Z4. n.t = not tested, n.c. = result not clear.

Beetle species	Morphological description of fungal colony	Source(s)	Growth rate (mm/day)	Mating with *O. piceae* mating types	Mating with other isolates from same beetle	Self-mating	Identification based on DNA analysis and morphology
T. retusum	brown with pesotum, typical *Ophiostoma*	larvae, pupae, females, galleries, mycangia	2.5 ± 0.2 (n = 12)	50% perithecia (n = 11)	50% perithecia (n = 18)	homothallic	*Ophiostoma piceae*
	large white fluff	mycangia	3.0 ± 01 (n = 10)	0% perithecia (n = 3)	0% perithecia (n = 10)	n.c.	*O. piceae* *O. piliferum*
	small white fluff	larvae, galleies	1.2 ± 0.1 (n = 8)	n.t	50% perithecia (n = 8)	homothallic	*O. piceae* *O. quercus*
T. betulae	brown with pesotum, typical *Ophiostoma*	larvae, pupae, females, males, mycangia	2.5 ± 0.2 (n = 7)	0% perithecia (n = 3)	0% perithecia (n = 13)	n.c.	*O. quercus* *O.* sp-2
	white fluff	larvae, female, mycangia	4.0 ± 0.4 (n = 10)	n.t	100% perithecia (n = 11)	homothallic	*O.* sp-1 *Ambrosiella* sp-1
T. lineatum	small white with small grey pesotum	mycangia	0.9 ± 0.1 (n = 6)	one with perithecia (n = 6)	100% perithecia (n = 8)	homothallic	*Ceratocystiopsis retusi* *O. quercus* *Ambrosiella ferrugine*
	large white	mycangia	1.6 ± 0.08 (n = 7)	n.t	100% perithecia	homothallic	*Ceratocystiopsis retusi* *O. quercus*

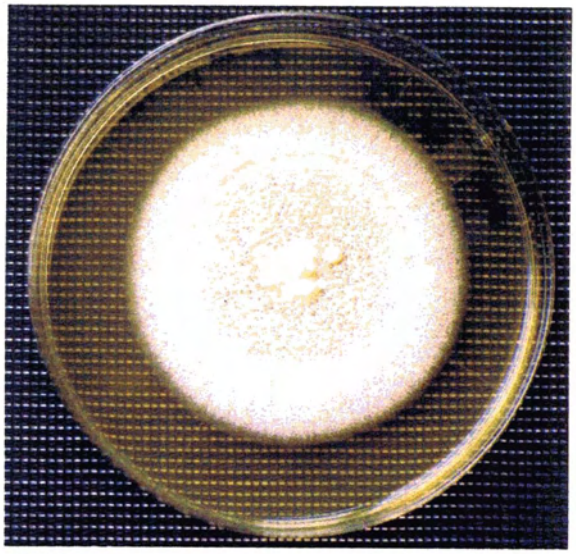
T. rufitarsus	large white fluff	mycangia, galleries	4.3 ± 0.2 (n = 7)	n.t	n.t	n.t	*Ambrosiella* sp-1 *Ambrosiella* sp-2 *Ceratocystiopsis minuta*-like
T. domesticum	brown with pesotum	mycangia	4.0 ± .01 (n = 8)	n.t	n.t	n.t	*O. piceae* *O.* sp-1 *O.* sp-2

Figure 3.1 Photographs of agar plates with representative fungal cultures of a) "small white fluff", b) "large white fluff " and c) "brown with pesotum anamorph".

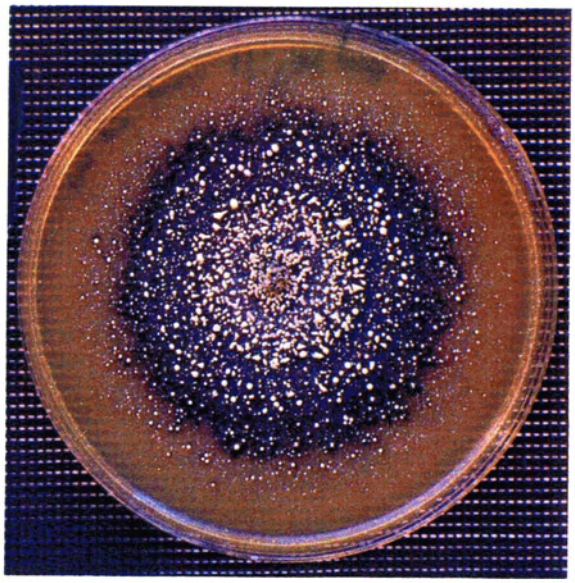
a)



b)



c)



the formation of perithecia teleomorphs in mating experiments or on subculture plates. The fungal growth forms varied markedly in their linear growth rates on agar media (Table 3.2). Comparison of morphological characteristics and growth rates combined with the DNA analysis indicated that three and (in one case) four different fungal isolates, most likely all distinct species, were consistently associated with each beetle species. *Ophiostoma piceae*, *O. quercus* (Georgev.) Nannf. and *O. piliferum* (Fries) H. & P. Sydow were isolated from *T. retusum*. *Ophiostoma quercus* and two other *Ophiostoma* species that were not further identified named *Ophiostoma* sp-1 and *Ophiostoma* sp-2, as well as an *Ambrosiella* species, *Ambrosiella* sp-1, were isolated from *T. betulae*. *Ophiostoma quercus*, *Ambrosiella ferruginea* (Math. Käärik) L. R. Batra and *Ceratocystiopsis retusi* (R. W. Davidson & T. E. Hinds) H. P. Upadhyay were isolated from *T. lineatum*. *Ambrosiella* sp-1, *Ambrosiella* sp-2 and a fungus that resembles *Ceratocystiopsis minuta*-like (Siemaszko) H. P. Upadhyay & W. B. Kendr. were isolated from *T. rufitarsus*. *Ophiostoma piceae*, *Ophiostoma* sp-1 and *Ophiostoma* sp-2 were isolated from *T. domesticum*. It is not yet clear if the species that are only identified to the generic level are unidentified new species. The low success of mating of isolates from one beetle with all others from the same beetle and the negative results from self mating do not entirely rule out the possibility that a species is homothallic. Only positive results allow for confident conclusions.

3.4.2 Inoculations in Freshly Cut Trembling Aspen Billets

Mean values (± SE) for water content in billets from the three experimental trees were 122.2 ± 0.1% dry weight and 57.8 ± 1.3% saturation. Samples from the inner heartwood and outer sapwood had similar values.

Cultures that grew from kernels placed on MEA plates before and after inoculation grew with the same shape and growth characteristics as the cultures of the original isolates, indicating that the inocula were viable and uncontaminated. No contamination or detectable colonization occurred from the ends of the logs. A reaction zone coloured dark green to brown with a dark yellow edge was visible on the bark up to approximately 4 cm in diameter surrounding the entrance of all drill holes including uninoculated controls. Stains in the wood associated with control drill holes were a faint brown in colour and extended only a short distance from the holes, far less than the stain surrounding inoculated holes. *Ophiostoma piliferum,* which grew fastest in laboratory cultures (Table 3.2), grew slowest in the wood (0.62 ± 1.0 mm/day longitudinal growth), while *O. quercus*, the slowest growing species in the laboratory cultures, grew at the same rate as *O. piceae* in the wood (0.81 ± 0.5 and 0.81 ± 0.7 mm/day longitudinal growth, respectively). The growth of treatment combinations AB, AC, and BC was not measured. Radial growth from the deep holes never extended beyond 1 cm into the wood, and there was no detectable tangential growth. There was little visible stain associated with shallow wounds, indicating that none of the fungi colonized the phloem. Recovery of the fungi used as a single inoculum was 66% (N=15), 33% (N= 18), 30% (N=30) for isolates A, B and C, respectively. No attempt was made to recover fungi from treatment combinations AB, AC and BC. Successful recovery was accomplished up to 6 cm from the inoculation point. On MEA plates, recovered isolates grew in the shape and growth characteristics as in the original cultures. No contaminating fungi were cultured.

3.4.3 Inoculations of Living Trees

Cultures that grew from pre-colonized wheat grains placed on MEA before and after inoculation grew with the same shape and growth characteristics as the cultures of the original isolates, indicting that they were viable and uncontaminated.

The timing of inoculation coincided with the first observation of attack by *T. retusum* on a living tree at the experimental site, providing inferential evidence that the inoculated trees should have been in a susceptible phase (Smalley et al., 1993). I observed one female *T. retusum* boring into an apparently healthy standing aspen tree on 18 April, 2001. After one day the female had excavated a hole and rested inside that hole with her abdomen or her head visible. For the next three days I periodically observed a male outside the female's hole continuously drumming her abdomen with his antennae, interrupted only by intervals every few minutes during which he raised his front legs onto the end of her abdomen and shook her in a sequence of pushing movements. It was impossible to determine if it was always the same male, but only one was present at a time. On the fourth day the male was inside the hole and frass was pushed outside the hole. The tree was ultimately attacked by seven pairs of *T. retusum* that season. I dissected five of those the seven galleries on 20 July, 2001 and found therein 0-4 eggs (mean 0.8), 2-12 larvae (mean 6.8), 1-16 pupae (mean 7.4), and 0-2 callow adults (mean 0.4). This tree had a few wilted branches and moderately sparse foliage.

The inoculated trees showed no sign of stress like wilt, dieback or discoloration of foliage, and had a full crown well into the second autumn after fungal inoculation. Within-treatment variation in the formation of lesions on the bark surface and stained wood at the inoculation points was at least as great as was the variation between

treatments. Several lesions per treatment were measured on the outside of the bark and were estimated to be 2-4 cm in diam. The colour changed from the light grey-green of a typical young aspen tree to a brownish green with a narrow yellow boundary. Typically the insides of the drilled holes were black except for the control trees, in which the inside was tan or light brown. The black coloration sometimes penetrated radially into the surrounding wood for a few millimetres, but the main discolouration extended longitudinally up to 9 cm in both directions, and only 1 cm tangentially. The wood from 5-20 mm in a longitudinal direction from an inoculation point was often greyish to dark brown, and appeared dead; beyond this tissue, the wood was brighter rusty yellowish Figure 3.2a shows a typical staining pattern on an aspen log inoculated with either fungus A, B, or C, here it is fungus A, *O. picea*, Figure 3.1b shows stain caused by mechanical damage in a control treatment, and Figure 3.1c shows stain from a natural attack by *T. retusum*. All three species of fungi grew from wood chips taken from both the discoloured wood in the sample tree discs and from wood adjacent to the discoloured area.

3.5 Discussion

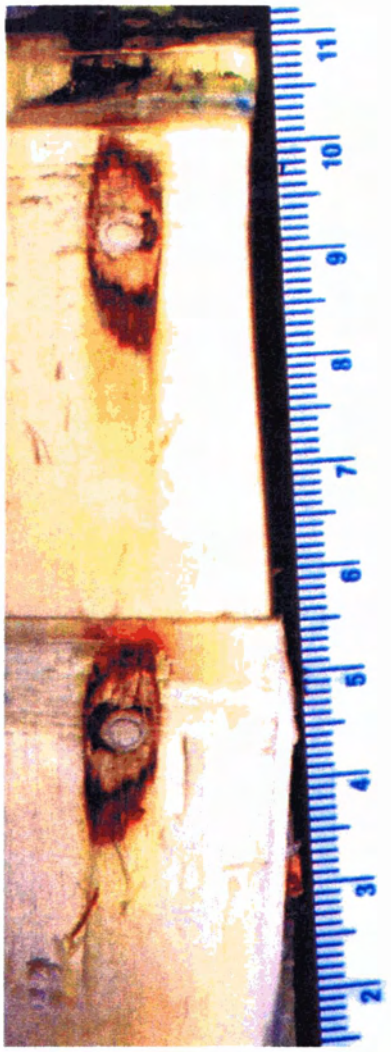
3.5.1 Isolations and Characterization of Fungi

My results reveal that each *Trypodendron* beetle species in B.C. is associated with at least three species of fungi. They indicate overlap in species-host association, but sufficient specificity in species complexes exists to potentially contribute to the reproductive isolation of their beetle hosts. To determine if any one beetle species could complete its' life cycle on the group of fungal species associated with any other species would have required labourious axenic rearing or antibiotic treatments

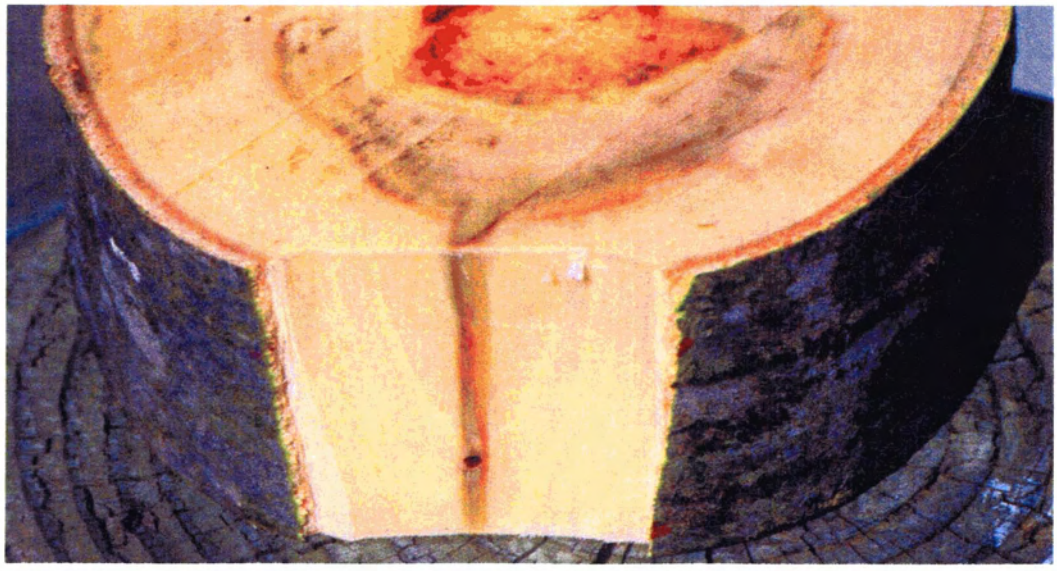
Figure 3.2 Photographs of staining patterns in wood of trembling aspen trees after inoculation of fungus A (*O. piceae)* (a), mechanical wounding in a control treatment (b), and a natural attack by a *T. retusum* (c).

a)

b)



c)



(Hunt and Borden, 1989) followed by introductions of authentic cultured fungi. It is also unclear if different fungi have different functions for the beetle, a question that could only be answered if microbe-free ambrosia beetles could be forced to rear their brood on only one introduced fungus.

Successful isolation was primarily dependent on the life history stage of the female beetle, especially isolations from mycangia. The mycangia of a post-reproductive female in a completed gallery system with no eggs appeared to be empty, as also described by Schedl (1964). The restriction of successful isolation only from females that were laying eggs could be explained by three possible hypotheses: 1) the fungus is not viable after the female has reproduced, 2) the mycangia are not replenished with fungi following reproduction, or 3) the fungi are inert and altered in some way that precludes germination and growth on standard media. Fungal vigour might be regulated according to seasonal changes in the activity of secretory glands and corresponding changes in activity of the beetle (Schneider and Rudinsky, 1969). Fatty oil produced by specialized hydrodermal cells or by glandular hairs (Baker, 1963) in mycangia just before hibernation in young adults (Batra, 1963) may deny the spores assess to oxygen. Together with other compounds that could be secreted into the mycangial lumen the oil may maintain the spores in a dormant-like state during hibernation, and reactivate them when growth and proliferation is required. The lack of contamination of isolates, especially when generated from the mycangia, is further indication of an active mechanism that eliminates all but the significant symbiotic fungi.

Francke-Grosmann (1958) described the mycangial tube, the type of sculpturing and the size and arrangement of the spines as species-specific forms in various

Trypodendron spp. For example, the mycangium of *T. lineatum* is a fine thin tube with isolated spines thinner and shorter than that of *T. domesticum* which also has especially thick sculptured pouches. Francke-Grosman (1958) did not state the beetles' life cycle stage at the time of fungus isolation, and some of her examples were from museum specimens or from overwintering beetles, neither of which allow for an interspecific comparison of fungal species. I found the size of the mycangium to be dependent on the size of the female, with the largest species, *T. retusum*, having the largest mycangia. The mycangia were also largest in beetles taken from their tree hosts early in the gallery excavation period.

Under natural conditions the fungi may never encounter freezing temperatures, because host trees are infected in the spring and summer and the beetles hibernate deep enough in the litter and duff to escape freezing in the winter. The corresponding lack of selection pressure to evolve freezing tolerance might explain why culturing was not successful after freezing the beetle. Alternatively the fungal spores might not be killed, but knowledge is lacking on how to reverse the effects of the cold temperature in laboratory cultures. Fungi that are closely associated with the insects may possibly require more complex or specific culture media and growing conditions than those less closely associated with insects hosts (Mathiesen-Käärik, 1960), which might explain why *O. piceae* and *O. quercus*, two common fungi on coniferous and deciduous trees, were frequently cultured.

A typical gallery of *Trypodendron* sp. darkens during offspring production to a deep brown to black in the main tunnel system as the walls get covered with dark hyphae, while a 'white fluffy' fungus grows in larval and pupal cradles. If the former fungus is

plated on media it turns brown and the hyphae blacken with age, as also observed by Baker (1963) and Francke-Grosman (1958), corresponding to the brown isolates of *T. retusum* and *T. betulae* (Table 3.2). The eggs of *Scolytoplatypus shogun* Blandford (Coleoptera: Platypodidae) develop in extremely pure white fungi also found in the mycangia (Nakashima and Iizuka, 1982). A fungus identified as *Ceratocystis retusi* (R.W. Davidson & T.E. Hinds) was isolated from *T. retusum,* and described as a white layer of ambrosia fungi found only inside the cradle and upon which the larvae feed (Hinds and Davidson, 1972). This fungus could be either *O. piliferum* or *O. quercus* (Table 3.2). After exposing and incubating a gallery or after the young adults leave, the walls of the cradles darkened, but still might have contained some 'white fluffy' fungal mass.

Franke-Grosmann (1958) illustrated these two growth forms for *Trypodendron* ssp. and species in the tribe Xyloborini. The white fluffy form might be most easily digested, or even required by the larvae, while the dark form may be better able to sustain adults in the main tunnel system. It may also contribute to maintaining a favourable microclimate, may spread more efficiently in the host tree to overcome residual resistance, or may be antagonistic to contaminants that enter the gallery system. One form might also alter the wood to optimise the growth of the second form.

Symbiotic fungi essential to an ambrosia beetle may not all be carried exclusively in the mycangia. Some might be passively disseminated on the exoskeleton. Alternatively, as with *Ophiostoma minus* (Hedgc.) Syd. & P. Syd. associated with the southern pine beetle, the beetles may carry the asexual form and phoretic mites may carry the sexual ascospores (Bridges and Moser, 1983; Moser and Marcia-Samano, 2000;

Moser and Roton, 1971; Moser et al., 1974). However, there is very little evidence of fungi transmitted by mites associated with ambrosia beetle galleries, although some mites are present on *Trypodendron* beetles (Borden, 1988). Dowding (1984) suggested that the relationship between the ophiostomatoid fungi and bark beetles is in fact a relationship between the fungi and fungus-feeding arthropods that live on the beetles. The role of the fungi carried by mites in the *Trypodendron*-fungi relationship is unknown.

Several factors have contributed to an incomplete understanding of the fungi associated with ambrosia beetles. Fungi in general exhibit only slight morphological differences between related species (Brasier and Kirk, 1993). Although the chemical environments of fungi in the host tree may differ, the physical environments within the host tree tissues are similar. Therefore, selection pressure for morphological changes might not be strong, and may act to conserve physical structures, while acting more upon physiological traits (Six and Paine, 1997). Hence it is not surprising than that several morphologically similar species of fungi associated with scolytid beetles were overlooked in the past, while DNA analysis provides a tool to separate species. Thus the *O. piceae*-1 and *O. piceae*-2, distinguished to be associated with *T. retusum* (Table 3.2) are only slightly morphologically distinct, but could have greater differences in physiological and biochemical characteristics. Furthermore, because the sexual states are sometimes difficult to produce in culture, and may be formed only at a certain time in the beetles' life cycle, often only asexual stages are identified as being associated with beetles. The ascomata (ascocarps) of *Ophiostoma* spp. are sometimes, and under certain conditions very small and may have been overlooked (Six and Paine, 1997). One growth form could be the pleomorphic form of another as in the three species reportedly associated with the

live-tree-attacking Columbian timber beetle, *Corthylus columbianus* Hopkins (Coleoptera: Scolytidae): *Ambrosiella xylebori* Brader ex von Arx & Hennebert, *Pichia* sp. and *Fusarium* sp. However, *A. xylebori* is probably the pleomorphic form of *F. solani*, which is associated with other tree-killing ambrosia beetles, *Xylosandrus compactus* (Eichoff.), *X. germanus* (Blandford), and *Xyleborus ferrugineus* F. (Beaver, 1989). The majority of ambrosia beetles, like *Trypodendron* spp., seem to be primitively polyphagous, with monophagy evolved rarely and probably secondarily as in *T. retusum* and *T. betulae* (Atkinson, 1988).

Ophiostoma piceae is a ubiquitous sapstain fungus and weak parasite on a wide variety of holarctic coniferous and hardwood trees. In Canada it is the most common in sapstain of conifer logs and lumber in sawmills (Seifert, 1993; Uzunovic et al., 1999). It is economically important because of its role in bluestaining timber, and possibly in contributing to wide-spread oak decline in central and eastern Europe, although the pathenogenicity of this species has yet to be established (Harrington, 1993). In addition, *O. piceae* is believed to be closely related to *O. ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, and it has been suggested that it may have been involved in their relatively sudden appearance in Europe during the 20th century (Brasier, 2001; Brasier and Kirk, 1993). It is also closely related to *O. quercus* which is almost exclusively found growing on hardwoods (Brasier and Kirk, 1993; Kim et al., 1999). *Ophiostoma piceae* is so commonly found on wood substrates that one might doubt its importance in the insect-fungus relationship. It might be an evolutionary vestige of an ancestral insect-fungal relationship and was an easily accessible fungus when beetles evolved from a xylophagous to a xylomycetophagous habit, because it is very substrate tolerant. It could

also be important in combination with other fungi. Alternatively it might be an omnipresent contaminant that is not used by the beetles but 'hitch hikes' a ride to a new host. Many *Ophiostoma* including *O. piceae*, *O. piliferum* and *O. quercus* are categorized as sapstain fungi (Gibbs, 1993; Harrington, 1993) although I have not observed the typical blue-stain characteristics.

Mathiese-Käärik (1960) and Franke-Grossman (1967) provided evidence in favour of the hypothesis that ambrosia fungi are highly specialized, supporting Hubbard's (1897) statement, that "So far as we yet know the food of each species of ambrosia beetle is limited to a certain kind of ambrosia, and only the most closely related species have the same food fungus." My results (Table 3.2) suggest that this specialization occurs not because each fungal species is exclusively associated with only one beetle species, but because each beetle has its unique fungal community. The natural food of the beetles and their larvae is probably a mutualistic microbial complex (Haanstad and Norris, 1985), although the proportions of the various fungi and bacteria in the diet may vary with stage of development of the beetles and the age of the gallery system (Batra, 1966). Ambrosia beetles that are host specific either live in an environment with very few host trees and attack the most abundant trees species, or habitually attack living trees. The high host specificity of the latter species is presumably associated with the necessity to overcome the host defenses and to attack and grow in living trees (Beaver, 1989). Only the two host specialists among the native B.C. *Trypodendron* species, *T. retusum* and *T. betulae* on aspens and on birches, respectively, have been observed to attack standing apparently healthy trees.

The regularity with which blue stain fungi in the genus *Ceratocystis* have been found in ambrosia beetle tunnels was originally taken as an indication of a close association with the beetles, even though there is no evidence for their being primary ambrosia fungi (Baker, 1963). My finding of *Leptographium* sp. (*Ophiostoma*) associated with *T. lineatum* and *T. rufitarsus* in a tree attacked the previous year by *D. ponderosae* suggests that this hypothesis can probably be rejected. Moreover, previous reports paid no attention to the presence of other beetles in the host trees. I found *Ceratocystiopsis retusi* only in association with *T. lineatum* although it was first identified as a specialized fungus on *T. retusum* (Hinds and Davidson, 1972). A study on the frequency of fungi on various beetles should give an indication of the significance of the association (Gibbs, 1993; Mathiesen-Käärik, 1953; Six and Bentz, 2003). Furthermore interpopulation differences in the frequency of fungal associates have been noted for several species of bark beetles (Six and Bentz, 2003; Viiri, 1997). In some diseases the *Ophiostoma* fungi involved did not prove to the causative agent, and in other examples they perhaps contributed only in a minor way to the disease symptoms (Harrington, 1993). Harrington (1993) also states that *Ophiostoma* fungi are not the primary inhabitants of well-developed mycangia with secretory cells in bark beetles. Thus, the significance of the *Ophiostoma* fungi associated with *Trypodendron* ambrosia beetles remains unclear.

3.5.2 Fungal Growth in Freshly Cut Billets of Trembling Aspen

My results showed that fungi isolated from *T. retusum* and their galleries are capable of colonizing the living tree tissue of freshly cut trembling aspen billets. The growth of all three fungi was most pronounced longitudinally following the alignment of

xylem vessels and was limited in other directions. This probably reflects in part the greater physical barriers to penetration in these directions (Gibbs, 1993). Pathogenic potential is suggested by the ability to grow from holes drilled 3 mm deep into living woody tissue, and by retrieval from the tree tissue, well removed from the point of inoculation, even in the absence of ambrosia beetle associates that normally tend the fungi. Based on the relatively limited extent of growth, the fungi appear to be weak pathogens that might kill a tree only when it is severely mass attacked. However "patch" kills associated with even single galleries would be consistent with field observations. These findings are in agreement with the ability of other ambrosia fungi to penetrate the wood adjacent to the tunnels, filling the cells, particularly the parenchyma, with a tangled mass of dark hyphae, and possibly exuding fruit esters that might be also responsible for the dark coloration of the wood (Neger, 1908a, 1908b, 1909). The relative lack of visible stain associated with shallow wounds, indicates that none of the fungi colonized the phloem tissue, reducing the potential for these fungi to girdle trees. In comparison with my results, the stain caused by fungi associated with *T. domesticum* in birch occurred only around the tunnels, while stain in conifers attacked by *T. lineatum* extended up to 2-3 cm into the sapwood (Bakshi, 1950).

The relative absence of stain around the aseptically drilled control holes demonstrates that staining associated with inoculated holes was due to the presence of microorganisms. The discolouration around the control holes is probably a wound response (Faulds, 1973) expressed by the oxidation of phenols that are subsequently polymerized to darkly-colored polyphenols (Shigo, 1965).

Althought moisture content of the billets was recorded, the role of varying moisture content was not studied. The moisture content of conifer wood colonized by blue stain fungi was substantially lower than in non-colonized regions(Uzunovic, 1996). The potential role of attending beetles in reducing host moisture content and enhancing the pathogenicity of its associated fungi has also not been investigated.

3.5.3 Fungal Growth in Standing Trees

The longitudinal growth in living trees of fungi isolated from *T. retusum* confirmed the observation of a similar pattern of growth in trembling aspen billets. In conifers, the growth pattern is somewhat different, with both radial and longitudinal mycelial growth rates being rapid, while the tangential growth rates are much slower (Langerberg et al. 1927 as cited in (Dowding, 1984). All three species of fungi were still present and alive six months after they were inoculated into the trees, but caused no wilt or any other visible indications of stress over two successive summers. Also no inoculated tree was attacked by *T. retusum*, which would have been a symptom of stress. The literature is somewhat equivocal on this point. Successful attack and brood development of the southern pine beetle and death of the host tree have been observed without blue stain fungi (Hetrick 1949). In contrast some authors claim that ambrosia fungi can not grow in the absence of the insect (Batra and Downing Michie, 1963; Meiffen and Belin, 1960). In support of this claim experiments in which young shoots of *Coffea* sp. were infected artificially with pure cultures of the ambrosia fungi of *X. compactus* Eichhoff gave negative results (Francke-Grosmann, 1958; Francke-Grosmann, 1967).

The inoculation density was estimated to simulate natural attack by *T. retusum*, and I assumed that the transferred inoculum exceeded the amount present in the mycangia of one beetle. However, the amount of fungus transferred in either case is difficult to estimate, because the females carry spores while the inoculum consisted mostly of mycelium. Doubling the number of inoculum points of the vascular stain pathogen, *Ophiostoma polonica* Siem. (is now placed in *Cerotocystis polonicum* (Siemaszko) C. Moreau), on Norway spruce produced an almost eight-fold increase in symptoms (Webber and Gibbs, 1989). The establishment of an infection is also dependent on the physiological condition of the fungus and the environment and on the effects of the competing microorganisms. However, lodgepole pines will survive a higher density of artificial fungal inoculation than natural beetle attack (Raffa and Berryman, 1982).

The rusty yellow colour underneath the bark of lesions surrounding the points of inoculation may indicate successful fungal colonization (Reid et al., 1967). Because stained wood extended the same distance below the wounds as above, the upward movement of water did not have an apparent effect on fungal growth. Although sapwood can appear stained after mechanical damage alone (Gries and McManus, 1965), and there was some discolouration of the control trees that were mechanically wounded, the recovery of fungi from more strongly discoloured areas of the sapwood from inoculated trees suggests that the stain in inoculated trees was a result of the fungal invasion, instead of a wound response of the tree. Resistance barriers such as starch-filled parenchyma or swollen ray parenchyma cells are stimulated by phytoalexins, and can be part of induced resistence to fungal invasions, e.g. in elms attacked by *O. ulmi* (Smalley et al., 1993). It is

possible that a similar induced resistance limited fungal growth in the inoculated trees. In any case the production of brood by *T. retusum* in the naturally attacked tree suggest that successful attack of living trees requires fungal invasion to extend only slightly beyond the beetles' gallery system.

Restriction of water transport by parenchyma ray cells caused by fungi associated with the mountain pine beetle can result in desiccation of sapwood (Reid, 1961; Yamaoka et al., 1990). Slight changes in water relations caused by fungal infection in one year may cause the death of a tree in the following year. I have observed other trembling aspens that were dead in the year following mass-attack by *T. retusum*, and it was most likely fungal infection that killed the trees (Solheim et al., 1993). I suspect that the tree killing capacity of the fungus might be contingent on the participation of the beetle. The beetles influence the condition of both the fungus and the host tree and boring activity is an effective way of spreading the spores and or hyphae over a large area.

If a tree is below the optimal water status, because of wind throw, root disease, drought or suppression by neighbors, or if supply of photosynthate to the bole is inadequate because of defoliation, water shortage, toxins, or shading, tree's resistance mechanism may weaken or fail entirely (Dowding, 1969). Forestry activities that promote a high density of suppressed and drought-susceptible trees, may lead to future increases in attack of standing trees.

4 Evolution of Reproductive Isolating Mechanisms in *Trypodendron* Species

4.1 Introduction

Reproductive isolating mechanisms are biological properties that prevent interbreeding of natural populations of closely related species. In insects, reproductive isolation is often mediated by volatile or contact pheromones, which are used as signals to attract conspecifics for mating or for resource exploitation (Coyne et al., 1994; Inomata, 2000; Roelofs and Cardé, 1974; Tumlinson et al., 1974; Via et al., 2000). Pheromones consist of either single compounds, or more commonly blends of compounds and typically show high species-specificity (Roelofs, 1995). Species-specificity is thought to have evolved because of the high costs of interspecific courtship and mating and lowered fitness through hybridization (Dobzhansky, 1940). Mate attracting signals of closely related, sympatric and syntopic (same host) species should be more divergent than those of closely related allopatric and allotopic (different hosts) species as a result of reinforcement and character displacement (Butlin, 1987; Butlin, 1995; Coyne and Orr, 1997; Rivas, 1964). This effect should be found not only when two species evolve in sympatry but also when species are already reproductively isolated and become sympatric (Butlin, 1987). However, if species are not capable of hybridization, reinforcement and character displacement are not necessary and interspecific competition may shape the divergent evolution of pheromones among sympatric species (Raffa, 2001).

Regardless of the mechanism, the high species-specificity of pheromones among scolytid beetles suggests that a selection process exists against small modifications in these signals. Thus, gradual evolution of pheromones caused by small modifications in

the chemical structure of respective compounds or their relative quantities in a mixture may be unlikely. Sudden, major (saltational) shifts to very different chemical phenotypes are perhaps more plausible (Baker, 2002; Roelofs et al., 2002; Symonds and Elgar, 2003).

However, small enantiomeric shifts probably occurred in the divergence of two sympatric sibling *Gnathotrichus* spp. One species, *G. sulcatus* Le Conte, utilizes a 65:35 blend of (*S*)-(+)- and (*R*)-(-)-sulcatol, and does not respond to either enantiomer alone. The sister species, *G. retusus* LeConte, produces and responds only to (*S*)-(+)-sulcatol and is repelled by the antipode (Borden et al., 1980a). Reproductive isolation through gradual shifts in enantiomeric ratios may occur in species with wide geographic ranges, within which there may exist differential interspecific competition for communication channels. Thus, pine engravers, *Ips pini* Say, in eastern North America utilize a 65:35 blend of (*S*)-(+) and (*R*)-(-)-ipsdienol, whereas blends of western populations have decreasing amounts of the (*S*)-(+) enantiomer, culminating in California, where almost pure (*R*)-(-)-ipsdienol is required to establish a separate communication channel from that of the California fivespined ips, *I. paraconfusus* Lanier, which uses the antipode (Birch et al., 1980; Lanier et al., 1980). Moreover, eastern populations of *I. pini* use an extra component, lanierone, in a pheromone blend not utilized to the same extent by western populations (Seybold et al., 1995; Teale et al., 1991). However, despite evidence to the contrary, Symonds and Elgar (2003) regard pheromone components that differ in chirality and stereochemistry as being the same.

In either scenario, the pattern of these chemical phenotypes might parallel speciation events. For example, the pheromone communication systems of seven *Ips* species seem to be analogous to the beetles' phylogenetic relationships (Cane et al.,

1990). Other studies, however, suggest that no correlation exists between pheromone chemistry and bark beetle phylogeny (Cognato et al., 1997; Francke et al., 1995). For example, there was no evidence for parallel evolution of aggregation pheromone blends with speciation among *Dendroctonus* and *Ips* species (Symonds and Elgar, 2003; Symonds and Elgar, 2004).

The presence of particular species-specific semiochemicals may also be associated with host choice, as pheromone components are often metabolic derivatives of host tree chemicals (Landolt and Phillips, 1997; Renwick et al., 1976; Seybold et al., 2000). Therefore, species with overlapping host ranges and metabolic capability may have no option but to use similar pheromones, contradicting the predicted outcomes of reproductive character displacement and reinforcement. In the Scolytidae, host volatiles are often used as signals for primary attraction and in combination with pheromones can display synergistic, attractive effects (Borden, 1985; Byers, 1989; Seybold et al., 2000). Among phytophagous insects, including ambrosia beetles, generic-level sympatric divergence is driven by host shifts (Drès and Mallet, 2002; Farrell et al., 2001; Linn et al., 2003; Marvaldi et al., 2002; Sequeira et al., 2000), which might be reflected in the relatedness of host chemicals used by these insects.

In termites and ants, cultivation of fungi has evolved once in each group, while molecular evidence suggests multiple origins of fungal cultivation in ambrosia beetles (Farrell et al., 2001; Mueller and Gerardo, 2002). Given the diversity of ambrosia beetle species (Farrell, 1998), such multiple origins might be expected. Also the wide variety of structures and sites of mycangia (Beaver, 1989; Francke-Grosmann, 1956) points to a polyphyletic origin of the ambrosia cultivating habits in the Scolytidae. The close

association of ambrosia beetles with their fungal partners might contribute to the reproductive isolation of sympatric species by e.g. transmitting specie-specific semiochemicals produced by the fungi.

Because of their direct feeding on fungi, that can utilize a broad range of host trees, ambrosia beetles are often considered host tree generalists (Beaver, 1989; Francke-Grosmann, 1967). However, two host tree specialists, *T. retusum* and *T. betulae* on *Populus* and *Betula* spp., respectively, may follow the trend of phytophagous insects in general toward increased host specialization (Crespi and Sandoval, 2000; Kelley and Farrell, 1998; Nosil, 2002). Models of diversification *via* adaptive radiation postulate the evolution of specialists from generalist ancestors as a consequence of increasingly fine resource specialization (Grant and Grant, 1989; Mayer, 1942). In ambrosia beetles, this could in turn be linked to the specialization of fungal use.

Morphological characteristics alone are not diverse enough to infer patterns of phylogenetic relationships among *Trypodendron* species or their sister genera in the tribe Xyloterini. Based on documented host use (all conifers in its' range and occasionally angiosperms) and the only beetles with a holartic distribution (Table 4.1) I hypothesized that *T. lineatum* would be found in a basal position to all *Trypodendron* species, and that *T. rufitarsus* is closely related to *T. lineatum*, given the pheromone and kairomone response and overlap in host use. I also predicted that the angiosperm attackers would be more closely related to each other than to the conifer attacking beetles.

The genus *Trypodendron* is thought to have originated in Eurasia and has apparently relatively recently reached North America (Wood 1982). Within the tribe Xyloterini Lindemann (which includes *Trypodendron* spp.) the monobasic *Xyloterinus*

TABLE 4.1 Individual identity, dates, locations, and methods of collection of *Trypodendron, Xyloterinus* and *Indocryphalus* beetles included in DNA analyses. Each collection date indicates an individual collected beetle. If not otherwise indicated, the collection location is in Canada. *Trypodendron domesticum* is native to Europe but is introduced and established in Canada.

Species	Collection date	Collection site	Method of collection
NORTH AMERICAN SPECIES			
T. lineatum	26 May, 2000	Brookmere, BC	pheromone trap
	26 May, 2000	Brookmere, BC	pheromone trap
T. rufitarsus	23 April, 1999	Brookmere, BC	pheromone trap
	23 April, 1999	Brookmere, BC	pheromone trap
T. retusum	04 June, 1998	Deep Gulch, BC	excised from aspen log
	1998	Brookmere, BC	excised from aspen log
	1998	Brookmere, BC	excised from aspen log
T. betulae	15 April, 1998	Mission, BC	excised from paper birch log
	15 April, 1998	Mission, BC	excised from paper birch log
T. domesticum	11 March, 1999	SFU campus, Burnaby, BC	pheromone trap
	12 March, 1999	SFU campus, Burnaby, BC	pheromone trap
	12 March, 1999	SFU campus, Burnaby, BC	pheromone trap
T. scabricollis [1]	March – April, 2003	State College, Pennsylvania, USA	pheromone trap
	March – April, 2003	State College, Pennsylvania, USA	pheromone trap
X. politus	15 April, 1999	Surrey, BC	walking on birch
	15 April, 1999	Surrey, BC	walking on birch
	15 April, 1999	Surrey, BC	walking on birch
EUROPEAN SPECIES			
T. lineatum	19 September, 2001	Moselle, France	pheromone trap
	5 May, 1999	Sollenau, Germany	pheromone trap
	5 May, 1999	Sollenau, Germany	pheromone trap
T. signatum	5 May, 1999	Austria	pheromone trap
	September, 2002	Göttingen, Germany	pheromone trap
T. domesticum	5 May, 1999	Merkenstein, Austria	pheromone trap
	September, 2002	Göttingen, Germany	pheromone trap
T. laeve [3]	5 May, 1999	Sollenau, Germany	pheromone trap
	5 May, 1999	Sollenau, Germany	pheromone trap
ASIAN SPECIES			
Indocryphalus pubipennis [2]	23 February, 1999	Kyushu, Mt. Tachibana, Japan	no information available

Swaine is scarcely separated from *Indocryphalus* Eggers and is also only a recent addition to the North America fauna (Wood, 1982b). The development of phylogenetic tree construction theory and methodology has created opportunities for testing evolutionary hypothesis about the sequence of character transformation (Farrell and Mitter, 1993; Frumhoff and Reeve, 1994; Miller and Wenzel, 1995), such as pheromone chemistry, host use, and symbiontic fungi association.

4.2 Objectives

The objective of this chapter was to infer evolutionary pattern of pheromone use, host associations and fungal symbionts using the phylogenetic relationship of the beetle in the genus *Trypodendron*. In addition I wanted to determine if host-plant specialization (as found for example, in *T. retusum* and *T. betulae*) is derived from host generalization, and to test whether the morphological separation of the genera *Xyloterinus, Indocryphalus*, and *Trypodendron* within the tribe Xyloterini (Wood, 1982b) is supported by a molecular phylogenetic analysis.

4.3 Methods

All molecular genetic research was done in the Crespi Laboratory, Department of Biological Sciences, Simon Fraser University, with the expert assistance of Christine Partent.

TABLE 4.2 Distributions, host ranges and primary pheromones of *Trypodendron, Xyloterinus* and *Indocryphalus* beetles included in DNA analyses. Host ranges are approximate, and unless otherwise noted, indicate that a species colonizes all members of a genus within its continental distribution. *T. domesticum* is native to Europe but is introduced and established in Canada.

Species	Distribution	Host habit description	Host range	Pheromones
NORTH AMERICAN SPECIES [1]				
T. lineatum	*holarctic*	conifer *generalist*	*Abies, Larix, Picea, Pinus, Pseudotsuga, Thuja, Tsuga, and rarely in Alnus, Betula, Malus, Juniperus*	*(+)-lineatin*
T. rufitarsus	nearctic	*Pinus* specialist	*Pinus contorta* var. *latifolia;* rarely in other *Pinus*[2]	(+)-lineatin
T. retusum	nearctic	poplar specialist	*Populus*	(+)-lineatin
T. betulae	nearctic	birch specialist	*Betula*	*ESR*- and *ZRR*-tirathol
T. domesticum	Europe (introduced)	angiosperm generalist	*Prunus, Betula, Acer*	(+)-lineatin and *ESR*-tirathol ?
T. scabricollis	Eastern USA	Pinus *specialist*	*Pinus banksiana, P. echinata, P. resinosa, P. taeda*	(+)-lineatin
X. politus	nearctic	broad generalist	*Acer, Alnus, Betula, Carya, Castanea, Fagus, Fraxinus, Quercus, Picea, Pinus, Tsuga, Ulmus*	(+)-lineatin
EUROPEAN SPECIES [1]				
T. lineatum	Europe	conifer generalist	*Abies, Cedrus, Larix, Picea, Pinus*	(+)-lineatin
T. signatum	Europe	angiosperm generalist	*Acer, Alnus, Betula, Fagus, Fraxinus, Populus, Tilia, Ulmus*	(+)-lineatin and *ESR*-tirathol ?
T. domesticum	Europe	angiosperm generalist	*Acer, Alnus, Betula, Fagus, Fraxinus, Carpinus, Quercus, Robinia, Sorbus, Prunus, Morus, Juglans*	(+)-lineatin and *ESR*-tirathol ?
T. laeve	Europe	*Pinus* specialist	*Pinus excelsa*	(+)-lineatin
ASIAN SPECIES [1]				
I. pubipennis	Asia	angiosperm generalist	*Benzoin, Cleyera, Fagus, Ficus, Hamamelis, Lindera, Machilius, Phyllanthus, Rhus, Zelkova*	No information available

[1] See Wood and Bright, 1992, [2] Personal observation; previous reports of other hosts could have mistaken this species for *T. lineatum*.

4.3.1 Sampling

Eight of 15 species in the genus *Trypodendron* (one species is only known from the fossil record) were collected. Two species, *T. lineatum* and *T. domesticum* were obtained from both North America Europe and are included twice in the analysis. *Xyloterinus politus* (Say), the only species of its genus and the only other genus in the tribe Xyloterini in North America (Wood, 1982b) was included in the analysis. Also included was *Indocryphalus pubipennis* (Blandford) one of the eight species in the third genus in the tribe world wide (Wood, 1982b). All beetles except *I. pubipennis* were frozen individually at -10°C after collection. Up to three individuals per species or continental population were included in the analysis. Voucher specimens are stored at the Pacific Forestry Centre, Natural Resources Canada, Victoria, B. C.

4.3.2. DNA Extraction

Total genomic DNA was extracted from individual beetles following the phenol-chloroform extraction protocol (Hillis et al., 1996). The whole specimen was used for DNA extraction. DNA from some individuals was extracted using the Dneasy DNA extraction kit for animal tissue (Qiagen, Valencia, CA, USA). DNA of *I. pubipennis* was extracted by H. Goto (Japan) provided by Bjarte Jordal, University of Bergen, Norway, to Andrea Sequeira, Department of Biology, Wellesley College, Wellesley, MA, USA, who in turn provided it to me.

4.3.3 Amplification and Sequencing

Polymerase chain reaction (PCR) and cycle sequencing were performed to obtain partial sequences of mitochondrial CO1 for 21 individuals using the primers 1718 and

2411 (Simon et al., 1994) and partial sequences of *Wingless* gene for 11 individuals using the primers 5'WG1 and 3'WG2 (Ober, 2002). The temperature profile consisted of 40 cycles, as follows: denaturing at 95°C for 30 sec, annealing at 47°C for 60 sec for COI and annealing at 50°C for *Wingless*, and extension at 72°C for 60 sec. The PCR products were processed using exonuclease I and shrimp alkaline phosphatase, and Big Dye Cycle Sequencing (Applied Biosystems, Foster City, CA, USA) was used to sequence fragments about 450 base pairs (bp) long for COI. The *Wingless* gene fragment sequenced was 609 bp long (including gaps), and five of the 11 *Wingless* fragments were only 109 bp long.

4.3.4 Phylogenetic Analysis

DNA sequences were aligned by eye in Se-Al (Rambaut, 1996) and also checked using Clustal X program (Thompson et al., 1997) for the *Wingless* alignment. Ambiguous sequence alignment positions of the *Wingless* sequence were not included in subsequent analysis. Phylogenetic analyses were performed by maximum parsimony (MP) (Swofford, 2000), and maximum likelihood (ML) (Felsenstein, 1981). MP and ML were performed using PAUP Version 4.0b10 (Swofford, 2000). For each search method mitochondrial and nuclear sequence data sets were analyzed separately and combined. For MP analyses, searches were performed using the heuristic method. A starting tree was obtained by the stepwise random addition of sequences with 10 trees held per addition. Optimization was performed by TBR branch-swapping. All substitutions were weighted equally and gaps were treated as missing data for the COI dataset and as new characters for the *Wingless* dataset. Support for branches under parsimony was assessed by bootstrapping analysis with 1000 replicates for MP.

The program MODELTEST Version 3.06 (Posada and Crandall, 1998; Posada and Crandall, 2001) determined GTR+I+G and HKY+G to be the most appropriate model of sequence evolution for COI and *Wingless* data sets respectively. These models were then used in all subsequent ML phylogenetic reconstructions. In the combined data set of for COI and *Wingless* the model used was HKY, a simpler model compatible with both genes sequence data (Ti/Tv =0.9967) and six-parameter instantaneous rate matrix estimated using maximum-likelihood for COI sequence data (Rmat = 1.9351 12.5904 4.9980 0.7312 23.7343). Among-site rates were assumed to be variable following shape parameter (γ = 2.6000) for COI and (γ = 0.2104) for *Wingless*. Support for branches was assessed by bootstrap analyses with 1000 replicates. All trees were rooted using the sequences of the heterogeneric species *X. politus* from Canada and *I. pubipennis* from Japan as outgroups.

I infer ancestral pattern of pheromone detection and use and host-tree association using maximum parsiomony based on the ML tree of the combined data set. Host specialists are defined here as species that only occur in one genera of host species. Beetle species that are associated with more then one genera of host species is classified as host generalist.

4.4 Results

4.4.1 Sequence Variation

Totals of 449 bp and 609 bp were included in the analysis for COI and *Wingless*, respectively. The COI dataset comprised 21 different haplotypes, whereas 10 of the 11 sequences obtained for the wingless gene represented different haplotypes. For COI, 176 sites were variable (39.2%), of which 72.2% was variation at the 3rd codon position.

Overall 91.5% (161 sites) of variable COI nucleotide sites were parsimony informative. *Wingless* had only 129 variable nucleotide sites (21.2%), and of these only 31.0% (40 sites) were parsimony informative. Across all sites AT richness was 64.1% for COI and 67.8% for *Wingless*. Within ingroup taxa, the maximum divergence was 18.3% for COI and 7.27% for *Wingless*, and the divergences of ingroup taxa from the outgroup ranged from 17.3 to 23.0% and 7.2 to 17.3% for COI and *Wingless* respectively. The DNA sequence data has been included with the beetle vouchers specimens at the Pacific Forestry Centre, Natural Resources Canada, Victoria, B.C., and will submitted to GenBank after the acceptance of a manuscript for publication.

4.4.2 Phylogenetic Analyses

Analysis of the COI gene provides good resolution at the tip and the base of the trees, while *Wingless* provides good resolution in the intermediate nodes for both ML (Figure 4.1) and MP (Figure 4.3), with higher bootstrap values overall for the combined trees. The tips of the trees are very well supported with bootstrap values of 95-100%, except in some analyses of *T. domesticum* from B.C. and Europe (with bootstrap from 54 to 100%). In general the analysis of the combined data sets of *Wingless* and COI (Figure 4.2, and 4.4) provided more resolved and well-supported estimates of the historic interrelationship of *Trypodendron* species than the analysis of either region alone (Figure 4.1, and 4.3). In the *Wingless* analysis only a few nodes are resolved. The MP analyses of the combined COI and *Wingless* tree (Figure 4.4) gave higher bootstrap values for a given node than the ML analysis (Figure 4.2). In the ML analysis of COI as well as in ML of the combined trees, *T. rufitarsus* is grouped with *T. laeve* while in the MP analysis

Figure 4.1 Phylogenetic trees of two separate analysis CO1 (ML tree, with bootstrap support values (a dash means no support), and *Wingless* (contains the best ML tree for the *Wingless* data, the bootstrap support values are indicated near the nodes).

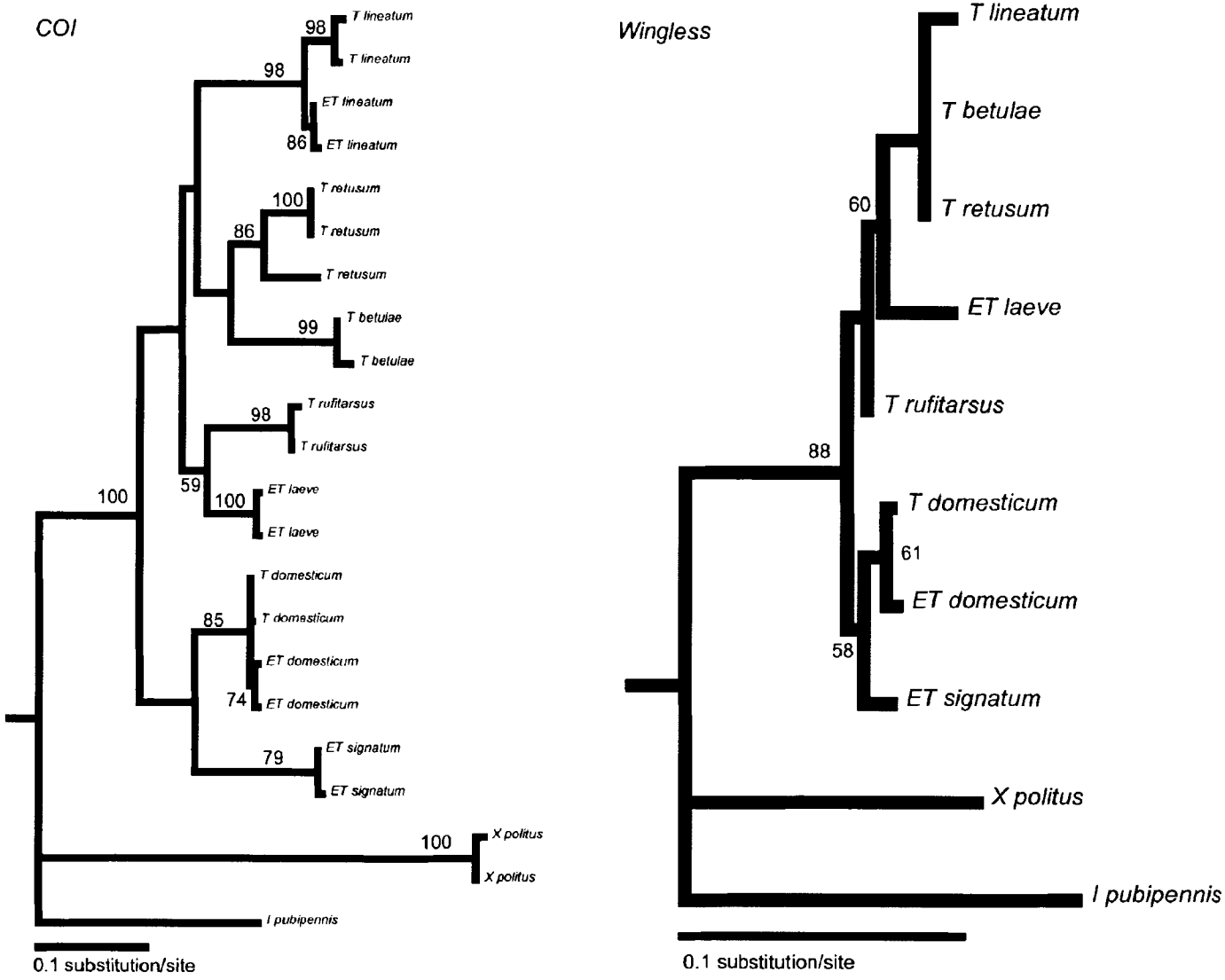

COI
T lineatum
T lineatum
ET lineatum
ET lineatum
T retusum
T retusum
T retusum
T betulae
T betulae
T rufitarsus
T rufitarsus
ET laeve
ET laeve
T domesticum
T domesticum
ET domesticum
ET domesticum
ET signatum
ET signatum
X politus
X politus
I pubipennis
98
98
86
100
86
99
98
59
100
100
85
74
79
100
0.1 substitution/site
Wingless
T lineatum
T betulae
T retusum
ET laeve
T rufitarsus
T domesticum
ET domesticum
ET signatum
X politus
I pubipennis
60
88
61
58
0.1 substitution/site

Figure 4.2 Phylogenetic tree of combined CO1 and *Wingless* (the best ML tree based on the combined datasets, and bootstrap support values indicated at the nodes).

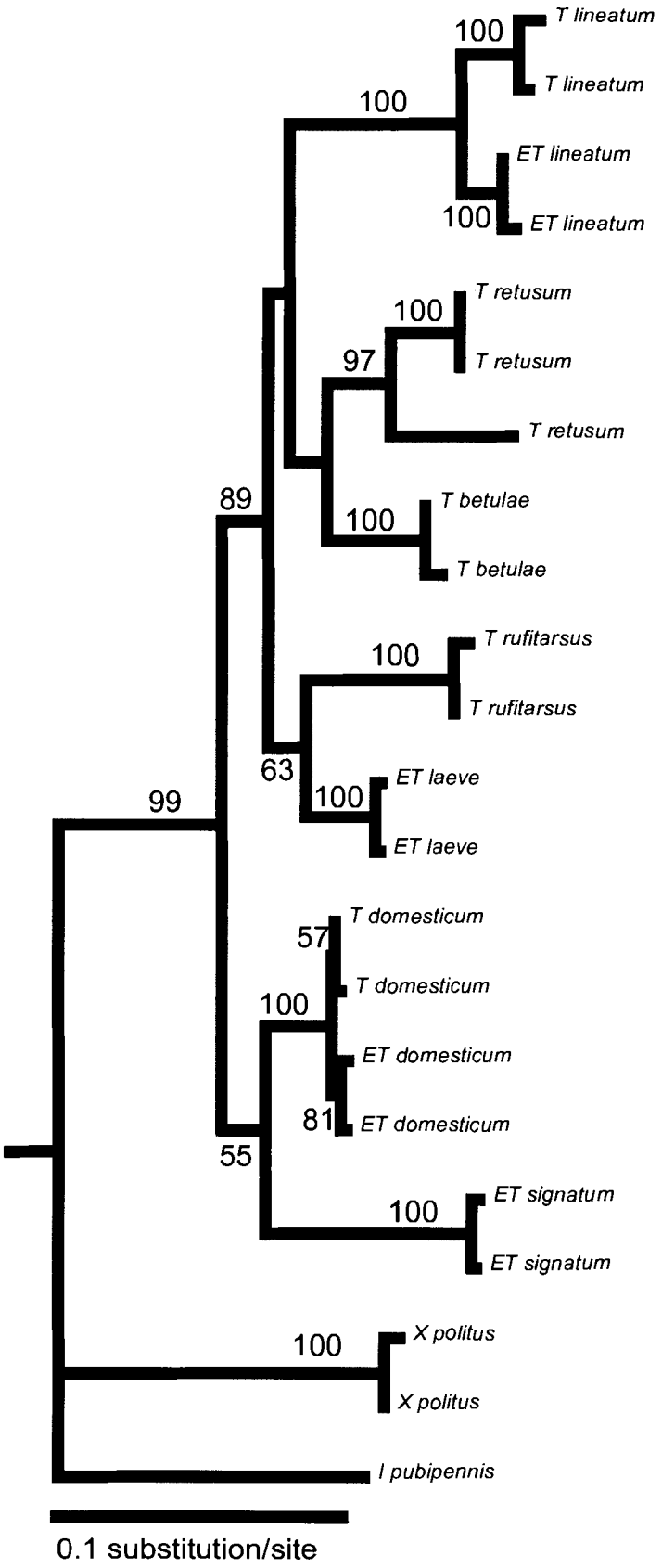
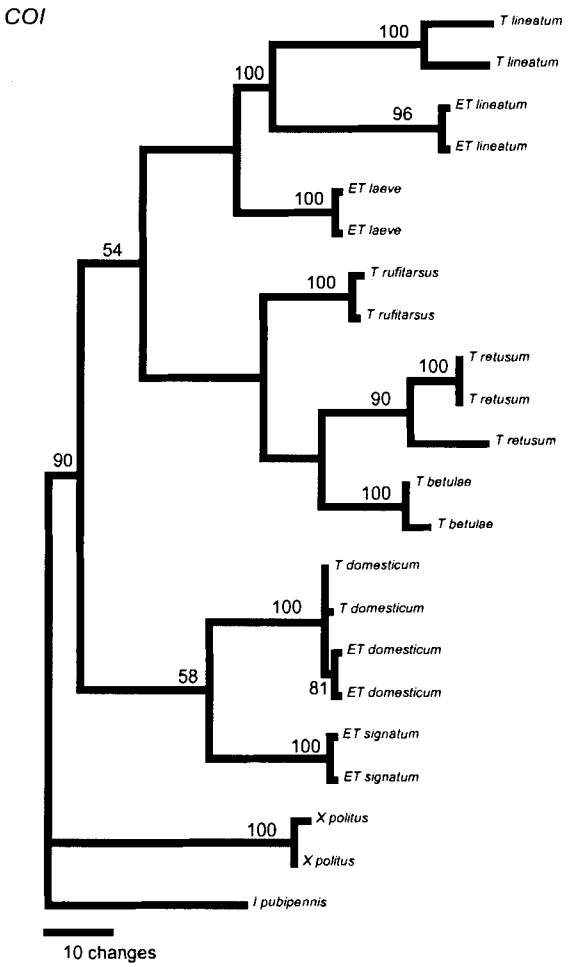

T lineatum
T lineatum
ET lineatum
ET lineatum
T retusum
T retusum
T retusum
T betulae
T betulae
T rufitarsus
T rufitarsus
ET laeve
ET laeve
T domesticum
T domesticum
ET domesticum
ET domesticum
ET signatum
ET signatum
X politus
X politus
I pubipennis
100
100
100
97
89
100
100
63
100
99
57
100
81
55
100
100
0.1 substitution/site

Figure 4.3 Phylogenetic trees of two separate analysis CO1 (MP tree, with bootstrap support values (a dash means no support), and *Wingless* (contains the best MP tree for the *Wingless* data, the bootstrap support values are indicated near the nodes).


COI
100
T lineatum
T lineatum
100
96
ET lineatum
ET lineatum
100
ET laeve
ET laeve
54
100
T rufitarsus
T rufitarsus
100
T retusum
90
T retusum
T retusum
90
100
T betulae
T betulae
T domesticum
100
T domesticum
ET domesticum
58
81
ET domesticum
ET signatum
100
ET signatum
100
X politus
X politus
I pubipennis
10 changes

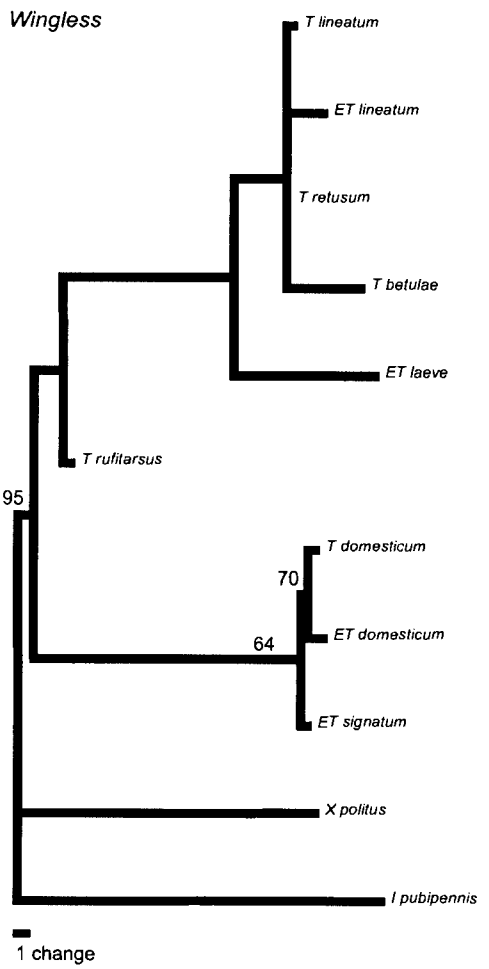
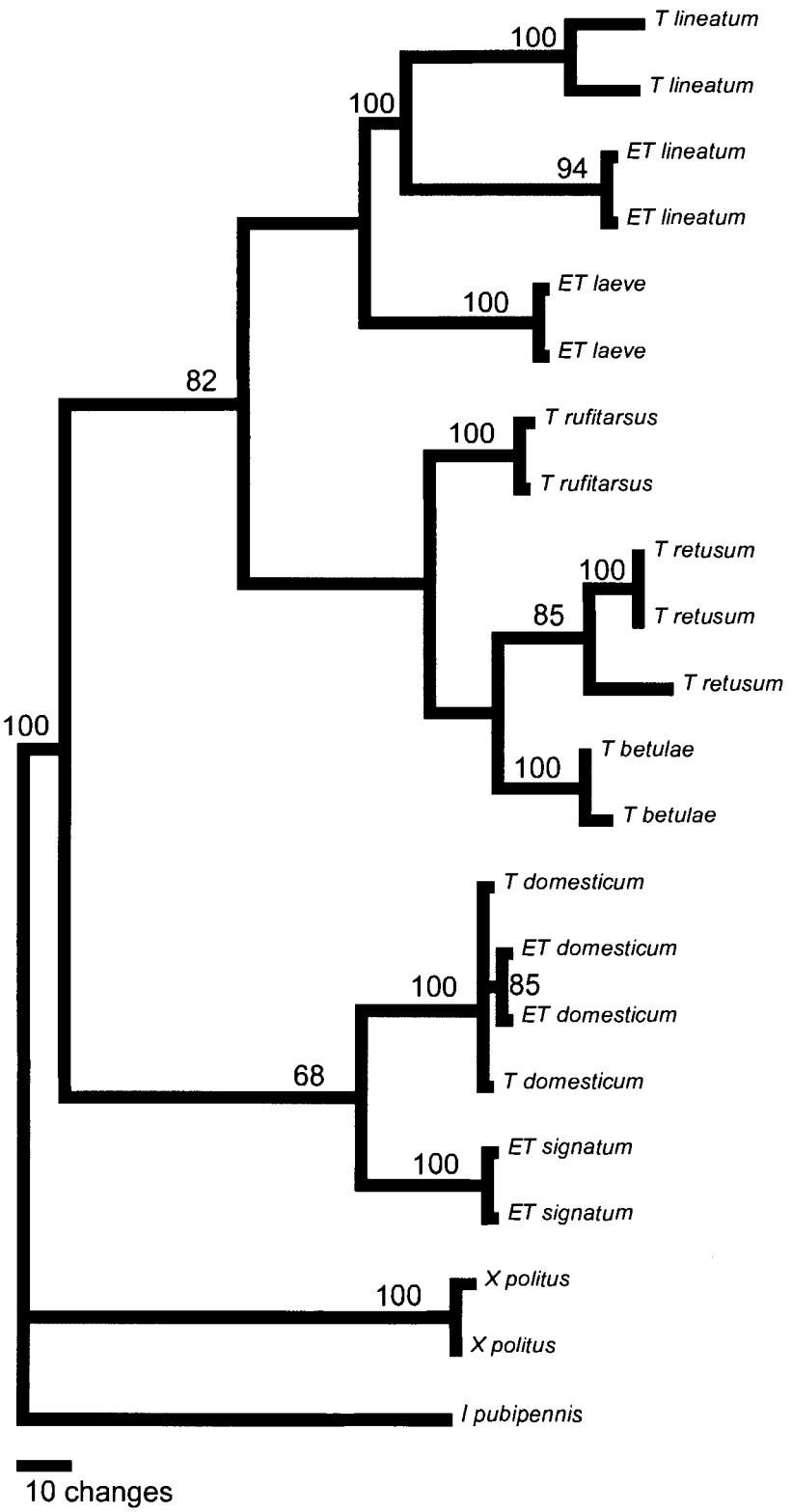

Wingless
T lineatum
ET lineatum
T retusum
T betulae
ET laeve
T rufitarsus
95
T domesticum
70
ET domesticum
64
ET signatum
X politus
I pubipennis
1 change

Figure 4.4 Phylogenetic tree of combined CO1 and *Wingless* (the best MP tree based on the combined datasets, and bootstrap support values indicated at the nodes).


T lineatum
T lineatum
ET lineatum
ET lineatum
ET laeve
ET laeve
T rufitarsus
T rufitarsus
T retusum
T retusum
T retusum
T betulae
T betulae
T domesticum
ET domesticum
ET domesticum
T domesticum
ET signatum
ET signatum
X politus
X politus
I pubipennis
100
100
94
100
82
100
100
85
100
100
100
85
68
100
100
100
10 changes

of COI it is placed with *T. lineatum* and in the MP analysis of the combined data it is placed closer to *T. lineatum*. There seem to be no differences between European and North American populations of either *T. lineatum* and *T. domesticum*, in any analysis. There is a strongly supported split from both gene regions and both analysis between *T. lineatum*, *T. laeve*, *T. retusum* and *T. betulae* on the one hand, and *T. signatum* and *T. domesticum* on the other. Finally, in all trees *T. signatum* and *T. domesticum* are sister taxa and basal to the rest of the *Trypodendron*.

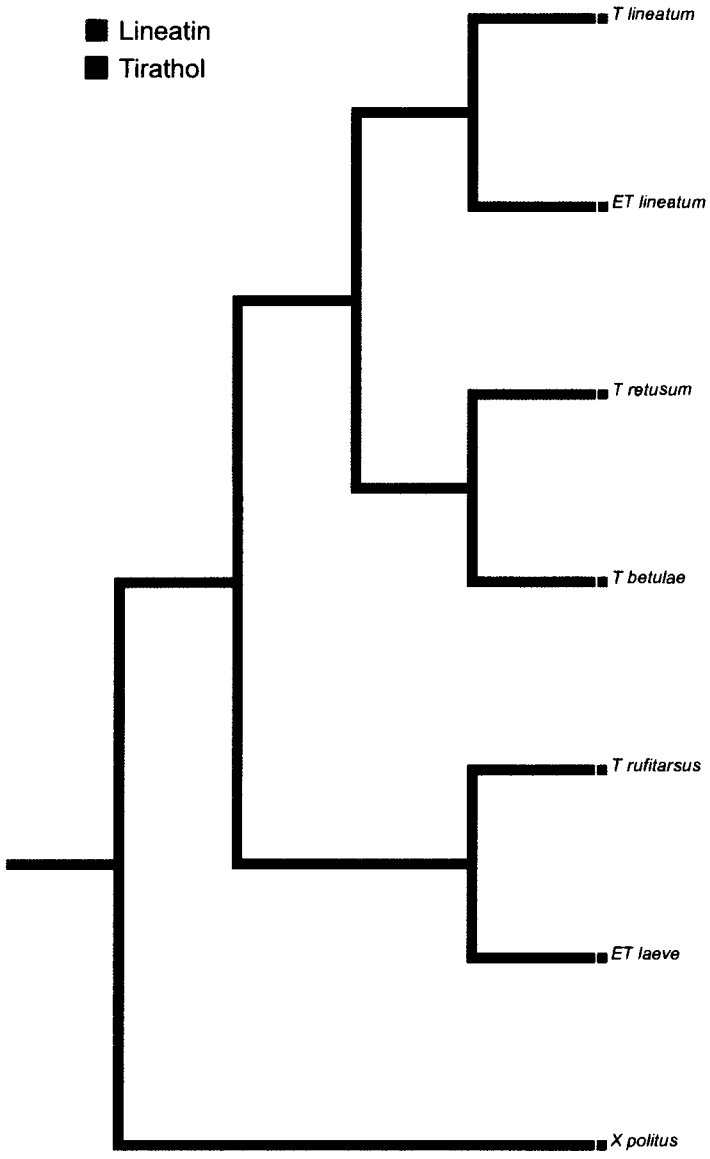
The use of the pheromone lineatin is most parsemoniously the ancestral state (Figure 4.5) in this group given that the outgroup species *X. politus* and all but *T. betulae* respond to it. Furthermore the sister taxa *T. signatum* and *T. domesticum* (basal to all other *Trypodendron*) can also detect and respond to one stereoisomer (*ESR*) of the second pheromone, tirathol. It is not clear yet if tirathol is used by *T. signatum* and *T. domesticum* in reproductive interactions.

The association with angiosperm host is the most parsimonious trait of an ancestor of this group (Figure 4.6). Specilization in the angiosperm host tree specialists *T. retusum* and *T. betulae* is, inferred as derived.

The molecular phylogenetic analysis supports the morphological separation of the genera *Xyloterinus, Indocryphalus*, and *Trypodendron* within the tribe Xyloterini (Wood, 1982b).

The phylogenetic analysis of the fungus species resulted in only a very weakly resolved and weakly supported tree and was not included here. The fungus species *O. quercus* is associated with three species related species. There is support that the

Figure 4.5 Most parsimonious reconstruction of ancestral pheromone production (on the left) and pheromone use (right), based on the best Maximum Likelihood tree of North American and European *Trypodendron* species.


Lineatin
Tirathol
T lineatum
ET lineatum
T retusum
T betulae
T rufitarsus
ET laeve
X politus

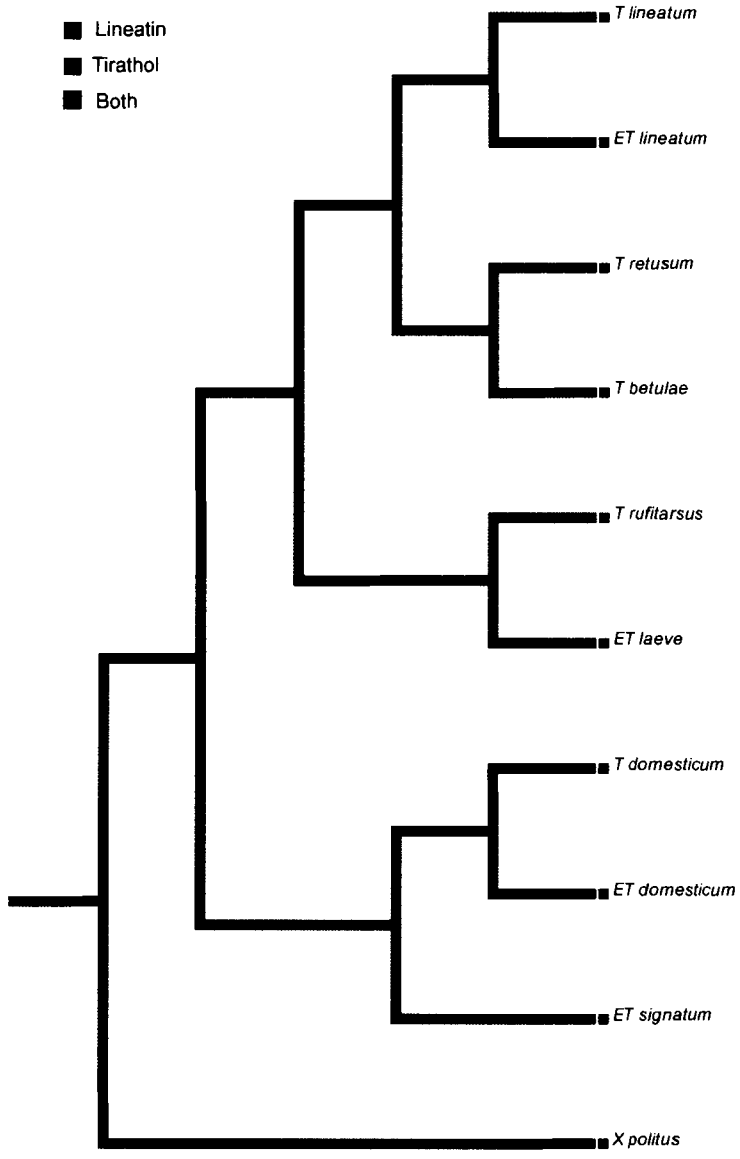
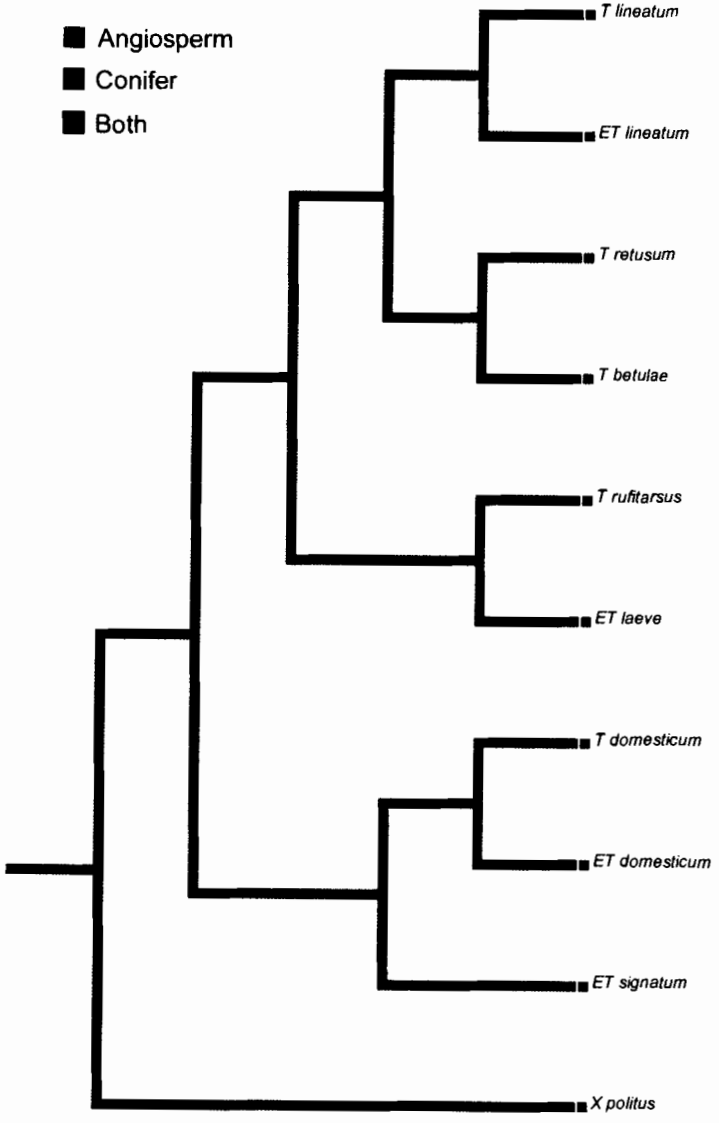
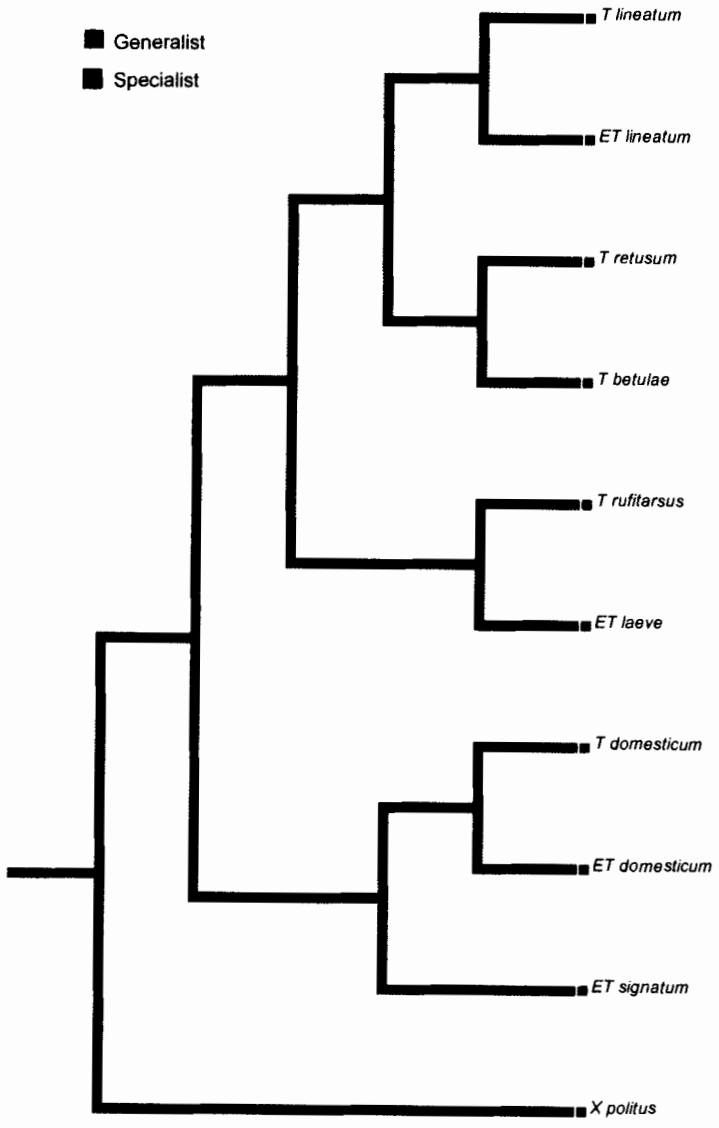

Lineatin
Tirathol
Both
T lineatum
ET lineatum
T retusum
T betulae
T rufitarsus
ET laeve
T domesticum
ET domesticum
ET signatum
X politus

Figure 4.6 Most parsimonious reconstruction of ancestral host association (on the left) and generalist and specialist (right), based on the best Maximum Likelihood tree of North American and European *Trypodendron* species. A Generalist is defined as species that is associated with hosts in the same genus (one or multiple species), while specialists are associated with multiple genera.


Angiosperm
Conifer
Both
T lineatum
ET lineatum
T retusum
T betulae
T rufitarsus
ET laeve
T domesticum
ET domesticum
ET signatum
X politus


Generalist
Specialist
T lineatum
ET lineatum
T retusum
T betulae
T rufitarsus
ET laeve
T domesticum
ET domesticum
ET signatum
X politus

haplotype found associated with the two sister taxa *T. betulae* and *T. retusum* is the same, but differs from the haplotyp of the fungus associated with the next closest species *T. lineatum*.

4.5 Discussion

My results validate the current assignment of species to genera in the tribe Xyloterini based on classical taxonomy, revealing phylogenetic relationships within the genus *Trypodendron*, and are valid phenotypic traits that distinguish *Trypodendron* species.

Greater confidence can be placed in the phylogenetic trees based on the combined data set than on the analysis of either gene section alone, because of characters from different sources informing the phylogenetic inference.

Only one species, *T. betulae*, included in this analysis uses a different pheromone, *ESR*- and *ZRR*-tirathol, than all other species, which use (+)-lineatin. Clearly, reproductive isolation within the genus *Trypodendron* and the tribe Xyloterini cannot be based on pheromone use alone, and the remarkably limited diversity of pheromones in this group does not permit inference about the reproductive isolation and speciation in relation to the evolution of chemical phenotypes as is possible for other taxa (Cardé, 1986; Cardé et al., 1977; Cognato et al., 1997; Coyne et al., 1994). Because *T. betulae* represents a recently-evolved species, tirathol use could be a new character replacing the ancestral use of the pheromone lineatin. Although the use of tirathol has only been demonstrated in *T. betulae*, European *T. domesticum* and *T. signatum* and *T. domesticum*

from B.C. did respond to one stereoisomer of tirathol (*ESR*) in GC-EAD analysis, and also responded to *ESR*- and *ZRR*-tirathol in one field trapping experiment (Chapter 2, and unpublished GC-EAD results by R. Gries, Simon Fraser University and J.-C. Grégoire, Universite Libre de Bruxelles). This either means that the response to tirathol or certain of its stereoisomers has evolved twice or that it evolved in a common ancestor of *T. betulae*, *T. domesticum* and *T. signatum* and was lost in all other *Trypodendron* spp. Because *T. betulae*, *T. domesticum* and *T. signatum* have overlapping host tree ranges, with *T. betulae* attacking birches and *T. domesticum* and *T. signatum* attacking various angiosperm trees, including birch, the response to tirathol could have developed, because it was produced as by-product from metabolism of the precursor linalooloxide, which is found in birch bark. The chemical structure of tirathol is not related to lineatin supporting the hypothesis of a major, saltational shift to a very different chemical phenotype (Baker, 2002; Roelofs et al., 2002; Symonds and Elgar, 2003).

The ancestral state analysis of pherpomone use in the European and North American *Trypodendron* species (Figure 4.5) indicate that the use of both together, and the production of lineatin is the most parsimonious, respectivley. The production of tirathol in *T. domesticum* and *T. signatum* is not known. That information would strengthen the analysis to infer the ancestral state of pheromone use.

In the ML tree of the combined data, *T. rufitarsus* and *T. laeve* emerge as sister species. There are remarkable similarities between *T. rufitarsus* in North America and *T. laeve* in Europe, with both flying very early in the season at temperatures well below the threshold for *T. lineatum*, with very short flying periods (Martikainen, 2000), and pers. obs). *Trypodendron lineatum* may attack the same tree as *T. rufitarsus* in B.C. and *T.*

lineatum the same tree as *T. laeve* in Europe (Martikainen, 2000). No observations on the overwintering habit of *T. rufitarsus* are reported, but it may have a similar habit as *T. laeve* which overwinters in the bark of standing tree. This habit would explain why both species can fly when the ground is covered with snow. Also both species have smaller conifer host ranges than *T. lineatum*, mainly species in the genus *Pinus*. These phenotypic similarities are consistent with the phylogeny.

The tribe Xyloterini consists of 23 species with 14 assigned to *Trypodendron*, eight to *Indocryphalus* and one to *Xyloterinus* (Wood and Bright, 1992). [The Asian *T. ashuensis* (Murayama) has been placed in synonomy with *Indocryphalus majus* (Eggers), *I. dainichiensis* (Murayama) is a synonym of *I. aceris* (Niijima), and *I. sinensis* (Eggers) is a synonym of *I. sordidus* (Blandford) (Bright and Skidmore, 2000), and *Trypodendron laeve* Eggers and *T. proximum* (Niisima) have been synonymized (Martikainen, 2000). *Trypodendron impressum* Scudder is only known from fossil records (Wood and Bright, 1992). With these taxonomic synonomies, the genera *Trypodendron* and *Indocryphalus* comprises 11 and 7 species, respectively]. Of these 63% inhabit angiosperms, 31% inhabit conifers and 6% live in both (Sequeira et al., 2000). The parsimonious ancestra state analysis of host use indicates ancestral association with angiosperms and switches to coniferous and back to angiosperm hosts within the tribe Xyloterini. This is consistent with the morphologically based clade that places the Scolytidae among the angiosperm feeding Curculionoidea (Wood and Bright, 1992; Wood, 1982b). However, fossil records (Schedl, 1947) and recent molecular phylogenetic analysis (Sequeira et al., 2000) support an ancestral association with conifer hosts for the scolytid clade (Sequeira et al., 2000). Regardless of the ancestral association my results suggest that some *Trypodendron*

species shifted to conifer hosts from an angiosperm-attacking habit. The strongest support for this hypothesis is the basal position of the two deciduous generalists, *T. signatum* and *T. domesticum,* and the derived position of the conifer-inhabiting generalists *T. lineatum*, *T. rufitarsus* and *T. laeve*. The still disputed pattern of host type association at the higher systematic hierarchy level makes it unclear if the apparent angiosperm association of the Xyloterini is an ancestral or a derived trait. Multiple shifts to use of angiosperms in Curculionoidea have been identified, each time associated with increases in weevil diversity and subsequent shifts of some species back to gymnosperms, particularly in the Curculionidae (Marvaldi et al., 2002). There is no evidence that the beetles co-speciated with their hosts, but host shifts might have increased the divergence of ambrosia beetles (Farrell, 1998). The derived position of *T. retusum* and *T. betulae* and their specialized host habits fit with the tendency for directional evolution towards increased specialization, in this case most probably from conifer generalist to angiosperm specialist.

There is no evidence that the five sympatric *Trypodendron* spp. co-speciated with the fungal symbionts that I isolated from the beetles species, due to a lack of enough informative molecular data of the fungal species. The overlap of the fungal association of *Ophiostoma quercus* in the closely related *T. betulae*, *T. retusum* and *T. lineatum*, though might be an indication of such a relationship between fungi and their beetle vectors. The fungal haplotype of *O. quercus* associated with sister taxa *T. betulae* and *T. retusum* is the same, while it differes from the *O. quercus* associated with next closest related beetles species *T. lineatum*.

The xylomycetophagous habit of ambrosia beetles occurs in at least 10 tribes of Scolytidae and has evolved seven times, often in a single genus within a tribe (Beaver, 1989; Farrell et al., 2001). Information on the diversity of fungal species that are apparently associated with ambrosia beetles should be interpreted with care, because of possible inter-population differences in fungal symbionts. Also the records of different fungal associations with a beetle species could be an artifact of varying isolation techniques by different authors, or varying times of isolation that sample different fungal partners at different times of the beetles' life cycle. Also, no investigations have been conducted into the functional significance of isolated fungi, which might not be symbiotic ambrosia but only loosely associated fungi that are not essential to completion of the beetles' life cycle.

5 Conclusion

My research supports only in part the hypothesis that semiochemical signals serve as key reproductive isolating mechanisms for phytophagous insects. Several pertinent results and conclusions enumerated below provide a basis for further studies of reproductive isolation mechanisms and may lead to more enlightend management of ambrosia beetles pests.

1. Among the four sympatric *Trypodendron* spp. native to B. C. only the birch attacking *T. betulae* uses a species-specific pheromone, *ESR*- and *ZRR*-tetrahydro-2,2,6-trimethyl-6-vinyl-2H-pyran-3-ol (*ESR*- and *ZRR*-tirathol). Response to this newly-identified pheromone is synergized by a combination of the host volatile conophthorin, found in high amounts in birch, and by ethanol, an indicator of stressed trees. This species-specific response to semiochemicals is sufficient to achieve reproductive isolation for *T. betulae*.
2. All other sympatric species use the pheromone (+)-lineatin, adding to the already established cadre of European species that use lineatin. *Trypodendron retusum* is isolated from the two conifer attacking species *T. lineatum* and *T. rufitarsus* by its response to a blend of lineatin and the host volatile salicylaldehyde, which is found in large amounts in its host *Populus* spp. A greater response to salicylaldehyde by coastal than interior populations may be an adaptive response that facilitates location of widely scattered poplars on the coast. Reciprocal repellency of salicylaldehyde to *T. lineatum* and *T. rufitarsus*, and by the conifer volatile *alpha*-pinene to *T. retusum* further isolates the angiosperm- and conifer-inhabiting species.

3. I could find no evidence for semiochemical-based reproductive isolation between the sympatric and syntopic species *T. lineatum* and *T. rufitarsus*, which I often found in adjacent galleries on the same host tree. Partial temporal isolation is apparently achieved by a lower 8°C temperature threshold for flight by *T. rufitarsus* than 15.5°C for *T. lineatum*, which allows the former species to find hosts and mate before the latter is active. Further isolation of *T. rufitarsus* may occur through additional unidentified close range post-landing mechanisms, e.g. contact pheromones, acoustic signals, or morphological incompatibility. The host and mate finding mechanisms among lineatin-producing *Trypodendron* spp. appear to be more complex than previously thought and may involve synergistic integration of different stimuli as recently shown for olfactory and visual signals in *T. lineatum*.
4. The remarkably limited diversity of pheromones among *Trypodendron* spp. does not permit any conclusion from a molecular phylogenetic tree about the evolution of pheromones as reproductive isolating mechanisms. However the ancestral *T. domesticum* can physiologically detect *ESR*-tirathol, one of the two stereoisomer of tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol used by derived *T. betulae*, an adaptation that either evolved independently or is a lost trait in all other species in the genus. This is a good example of how the combination of chemical ecological methods (GC-EAD and field bioassays) with phylogenetic analysis allows for interpretations not otherwise possible.
5. The fungal relationships of B.C.'s four native *Trypodendron* spp. appear to be more diverse and less species-specific than most authors allege. For each species

specificity lies in overlapping complexes of fungal species, with one or more species in common with the complexes associated with other beetles. This specificity is similar to that in multicomponent insect pheromones, in which compounds in common to numerous species occur in species-specific blends. The fungi isolated from *Trypodendron* spp. in B.C. belong to the genera *Ophiostoma*, *Ambrosiella*, and *Ceratocystiopsis*, and at least some of the species have pathogenic potential as seen in their ability to invade the tissues of freshly cut and standing trembling aspen.

6. Because of the complexity of ambrosia beetle-fungal interactions, isolation from different anatomic locations, insect life-cycle stages, and galleries at different stages of development can greatly vary the determination of the significance of isolated fungi for the beetles. Only if these factors are controlled with strict protocols can significant conclusions be drawn.
7. The phylogenetic analysis of North American and European *Trypodendron* beetles revealed no correlation with pheromone chemistry, host association or fungal complexes, indicating that these traits are secondary adaptations. The European angiosperm generalist *T. signatum* is ancestral to the rest of the group, and is most closely related to the sympatric and sometimes syntopic *T. domesticum*. In contrast, the North American angiosperm specialists *T. retusum* and *T. betulae* are derived from conifer-associated ancestors.
8. Prior to my study forest management practitioners paid no attention to the different *Trypodendron* species; *T. lineatum* was accepted as the single species causing damage to conifer wood. My observations build on those of a few other

researchers to suggest that *T. rufitarsus* is also an economically important species, responding to the same semiochemicals as *T. lineatum* and with an overlap (even co-occupancy) of host trees. Furthermore the increasing commercial importance of angiosperm trees will demand that pest management specialists pay more attention to *T. retusum*, *T. betulae* and *T. domesticum* in the future. New challenges like global warming and it's putative effects on insect plant interaction, e.g. recent wide spread attack of living trees by European ambrosia beetles previously assumed to be secondary species, requires a heightened understanding of these insects.

APPENDIX I

Secondary ambrosia beetles in apparently healthy trees: adaptations, potential causes and suggested research [2]

During the course of my research I observed numerous instances of native *Trypodendron retusum* and *T. betulae* attacking apparently healthy, living trees. At the same time, alarming reports emerged of an outbreak of *T. domesticum* and *T. signatum* attacking living trees, primarily beech, in western Europe. These events provoked me to explore the possible causes and potential outcomes of ambrosia beetles attacking living trees, and an apparent increase in its frequency and intensity. Although this exploration departed from the original goal of my thesis, I consider it to be part of my research, and I have thus included it as an Appendix to this thesis.

[2] Integrated Pest Management Reviews, vol.6, 209-219, 2001

Summary

Some ambrosia beetles are primary attackers of healthy, living trees, but in recent years normally secondary species have been increasingly observed attacking living trees, either as exotics or in their native geographic ranges. I identified five factors that could underlie an increasing prevalence of attack by secondary ambrosia beetles on living trees: 1) early flight before the host tree has recovered the ability to resist attack in the spring, possibly associated with climate change; 2) nutritional independence from the host that may enable ambrosia beetles to feed on ambrosia fungi that live on dead tissue in a living tree; 3) potentially pathogenic fungi that could become more pathogenic with climate change or through hybridization with exotic strains; 4) cryptic behavior that facilitates international transport and the establishment of exotic species and genotypes; and 5) a complex chemical ecology that enables secondary ambrosia beetles to locate stressed living trees that may temporarily appear to be suitable hosts for secondary beetles. I propose four avenues of research that will lead to an increased understanding of attack of living trees by ambrosia beetles, and may facilitate the implementation of effective pest management strategies and tactics: 1) intensive surveys, particularly for exotic beetle species and associated fungal strains; 2) molecular genetics studies that would facilitate the identification of known and new strains and genotypes, particularly of ambrosia fungi; 3) studies of the pathogenicity of ambrosia fungi as well as other fungi that could predispose trees to attack; and 4) investigations of the chemical ecology of tree-attacking species that could lead to new pest management tools and tactics.

Introduction

The evolution of herbivory, particularly on angiosperms, enhanced the rate of insect diversification, and produced nearly half of all insects (Farrell, 1998). Many herbivorous insect plant interactions also involve microbial mutualists, such as plant pathogenic fungi (Paine et al., 1997), which expand the capacity of insects to use plant resources (Hölldobler and Wilson, 1990; Mueller et al., 1998). The suitability and susceptibility of a host for feeding, breeding, and fungal cultivation is determined by insect, host, and fungal parameters, as well as environmental factors.

Wood-boring ambrosia beetles (Coleoptera: Scolytidae or Platypodidae) do not consume the wood of their tree or log hosts, but build gallery systems in which they cultivate fungi as larval food (Batra, 1966; Francke-Grosmann, 1967). The conventional view of ambrosia beetles' typical hosts is that of unhealthy, physiologically stressed, dying or dead trees, in forestry sometimes called unthrifty trees, or injured parts of living trees (Wood, 1982b). Many ambrosia beetles can also be classified as secondary beetles, attacking a dying or dead host that is already exploited by at least one, often more aggressive and tree killing, primary bark beetle (Furniss and Carolin, 1977). While competition among secondary bark beetles, or between primary and secondary beetles may be severe in the limited, two-dimensional phloem resource in weakened hosts (Rankin and Borden, 1991) ambrosia beetles may find such hosts relatively easy targets, because the plants' defense is compromised or lacking, and competition is minimal in the three-dimensional xylem habitat (Borden et al., 1981). Despite the customary view of ambrosia beetles as secondary invaders, the literature holds examples of some species that are preferential or obligatory attackers of living trees (Table 1). One principal

Table 1 Selected examples of ambrosia beetles attacking living trees.

Association with living trees	Species (Family)	Biological notes	Reference
Primary attacker	*Corthylus columbianus* Hopkins (Scolytidae)	Infests angiosperm trees in many genera, including *Acer*, *Platanus*, *Liriodendron*, *Tilia*, *Fagus*, *Ulmus*, *Betulae* and *Quercus*, in eastern U.S.A. Prefers vigorous living hosts. Causes significant economic degrade of timber.	Baker (1972), Nord and McManus (1972)
	Corthylus punctatissimus (Zimm.) (Scolytidae)	Breeds in variety of angiosperm trees and shrubs in eastern North America. Especially lethal to young sugar maple, *Acer saccharum*, because galleries girdle the stem near the root collar.	Finnegan (1967) Baker (1972)
	Platypus mutatus (= *sulcatus*) Chapius (Platypodidae)	Infests many deciduous trees including citrus and avocado. Galleries impede sap circulation.	Costilla and Venditti (1992)
	Platypus apicalis White *Tretoplatypus cavicepst* (Broun) *P. gracilis* Broun (Platypodidae)	Attack *Nothofagus fusca* (Hook. f) Oerst. in New Zealand. Staining of wood always associated with ambrosia fungi, which suppress growth of host.	Faulds (1973)
	Austroplatypus incompertus (Schedl) (Platypodidae)	Attacks living, healthy *Eucalyptus* spp. in Australia. Only eusocial species among bark and ambrosia beetles, with a 2 to 4 year cycle from egg to adult.	Kent and Simpson (1992)
Usually secondary attacker, but primary attack habit prevalent as exotic beetle	*Xyleborus dispar* (F.) (Scolytidae)	Almost all deciduous trees can be hosts. Currently severe pest of chestnuts in North America, and some deciduous species in native range.	Bright (1968) Wood (1982) Bhagwandin (1993)
	Xylosandrus crassiusculus (Motschulsky) (Scolytidae)	Native to tropical and subtropical Asia, attacks many living ornamentals and fruit trees in non-native range (e.g. USA, Africa). Saplings killed by girdling.	Kovach and Gorsuch (1985) Wood (1982)
	Xylosandrus ferrugineus (F.) (Scolytidae)	In native environment (North and South America, tropical Africa) attack of living hosts rare, but hastens or causes death of host. Considered the most destructive scolytid in tropical areas. Over 180, mostly deciduous, host species.	Wood (1982)

Association with living trees	Species (Family)	Biological notes	Reference
Usually secondary attacker, primary attack habit apparently increasing	*Trypodendron domesticum* (L.) (Scolytidae)	Recently found in apparent outbreak on living, healthy common beech, *Fragus sylvatica*, in Belgium and other European countries. Also found attacking paper birch, *Betulae papyrifera*, and red alder, *Alnus rubra*, as exotic species in B.C.	L. H. Humble (pers.comm.); S. Kühnholz (unpubl. obs.) Gregoiré et al. (2003)
	Trypodendron signatum (F.) (Scolytidae)	Recently found associated with *T. domesticum* in outbreak on living common beech in Europe.	Grégoire et al. (2003)
	Trypodendron betulae Swaine (Scolytidae)	Frequently found in B.C. attacking apparently healthy paper birch, *Betula papyrifera.*	S. Kühnholz (unpubl. obs.)
	Trypodendron retusum (LeConte) (Scolytidae)	Commonly found attacking living, apparently healthy aspen, Populus tremuloides, as well as damaged and broken stems. Creates patch "kills" on otherwise healthy trees.	S. Kühnholz and J. H.Borden (unpubl. obs.)
	Platypus quercivorus (Murayama) (Platypodidae)	Recently found to attack *Quercus* spp. trees associated with Raffaelea sp. fungus as the cause of the oak dieback in Japan.	Kamata (2002)

difference in aggressiveness between bark and ambrosia beetles is the relative absence among ambrosia beetles of species that build up large populations in weakened hosts and then attack living trees. In recent years, however, there have been reports and observations of normally secondary ambrosia beetles attacking apparently healthy, standing trees, and possibly causing the trees' death. In this paper I provide a non-inclusive overview of ambrosia beetles that are reported to attack living hosts, and then discuss adaptations that may facilitate an increasing prevalence of attack on living trees, with particular emphasis on *Trypodendron retusum* (LeConte) on trembling aspen, *Populus tremuloides* Michx., in British Columbia (B.C.), Canada. Finally, I propose avenues for research that could lead to increased understanding of, and management capacity for tree-infesting ambrosia beetles.

Ambrosia beetles in living hosts

Among the few ambrosia beetles that are primary attackers of living trees (Table 1) are species such as the Columbian timber beetle, *Corthylus columbianus* Hopkins (Scolytidae), *Megaplatypus* [=*Platypus*] *mutatus* (Chapius) [=*Platypus sulcatus* Chapius] (Platypodidae), and the only eusocial species among bark and ambrosia beetles, *Austroplatypus incompertus* (Schedl) (Platypodidae). All preferentially attack healthy angiosperm tree species.

One example of a species that normally attacks weakened or dead hosts in its native habitat, is the European *Xyleborus dispar* (F.) (Coleoptera: Scolytidae). However, as an exotic in North America, this "shothole borer" is, like other non-native species, considerably more aggressive. The hosts associated with *X. dispar* include almost all angiosperm fruit and forest trees; it has also been infrequently collected from conifers

(Bright, 1968; Wood, 1982b). Attacks are normally limited to hosts that are physiologically compromised due to recent injury or stress. In an epidemic or outbreak situation, however, *X. dispar* may attack healthy trees in close proximity to stressed and limited, preferred host material. The beetle was probably introduced to the USA prior to 1816, and first officially reported by Peck in 1817 as *Anisandrus dispar* (Wood, 1982b), and recorded in California attacking a variety of healthy deciduous trees in 1924 (Linsley and MacLeod, 1942). It is of great concern in many fruit and chestnut-growing areas throughout the northwestern USA and western Canada where it is a serious pest of young chestnut trees, and may be the limiting factor in successful orchard establishment. Chestnut growers in the Pacific Northwest hold a new mass attack by *X. dispar* responsible for the ultimate demise of seemingly healthy trees (Bhagwandin, 1993). In addition to the attack of healthy trees in non-native environments, *X. dispar* has been found recently in it's native range in Austria to, sometimes massively, attack sycamore maple, *Acer pseudoplantanus* L., cherries, *Prunus* spp., common ash, *Fraxinus excelsior* L., and red oak, *Quercus borealis* Michx.f. Young trees, up to 4 m high were killed, especially those in which fungi were detected (Perny, 1997), but it was not affirmed if there was an association between the beetles and the pathogenic fungi.

Another example of an exotic xyleborinie ambrosia beetle that attacks living trees is *Xylosandrus crassiusculus* (Motschulsky). Native to tropical and subtropical Asia, where it occurs in a wide variety of hosts, it was first recorded from the continental USA in South Carolina (Anderson, 1974). It is now distributed throughout the Southeastern USA, equatorial Africa, and many regions in Asia, the South Pacific and Hawaii. It is a serious pest of ornamentals and fruit trees, including peach, plum, cherry, persimmon,

golden rain tree, sweetgum, shumard oak, Chinese elm, sweet potato, and magnolia (Chapin and Oliver, 1986; Deyrup and Atkinson, 1987; Kovach and Gorsuch, 1985; Wood, 1982b). A large number of attacks apparently result in death of the trees, especially of saplings, by girdling.

The genus *Trypodendron* in North America and Europe includes both host generalists and specialists. The host range for the holarctic *T. lineatum* (Olivier) includes most conifers, and that of the nearctic *T. rufitarsus* (Kirby) comprises all pine trees in its range (Wood, 1982b). In contrast *T. betulae* Swaine attacks only birches, *Betula lenta* L. and *B. papyifera* Marsham, and *T. retusum* is reported only on *Populus* spp., especially *P. tremuloides* (Wood, 1982b). All beetles of this genus are described as attacking only unthrifty host trees or logs (Wood, 1982b). My recent observations in the southern interior of B.C. may change this perception. I noticed attack by *T. retusum*, not only on wind-broken trees, and trees damaged by vertebrates or lightning, but also on apparently healthy, standing aspens of various sizes. The attack was sometimes by a single mating pair, but more often up to 20 or more pairs per tree. Some trees showed signs of wilting in the year of attack, while others were found dying in subsequent years. I also found standing paper birch, with no visible signs of stress, attacked by *T. betulae*. In both cases the beetles have co-evolved with their hosts, unlike *X. dispar* and *X. crassiusculus*, which exploited novel host opportunities in a new environment. The behavior of *T. retusum* and T. *betulae* is similar to that of *T. signatum* (F.) and *T. domesticum* (L.) that have been reported attacking apparently healthy native common beeches, *Fragus sylvatica* L., in Belgium and other European countries in 2001 (Grégoire et al., in press). Also L. M. Humble (Pacific Forestry Centre, Canadian Forest Service, pers. comm.) has

found exotic *T. domesticum* successfully attacking red alder, *Alnus rubra* Bong., and reproducing in standing paper birch, observations that I confirmed in 2001. These recent observations suggest that angiosperm-infesting *Typodendron* spp. may be expanding their host range to living, apparently healthy trees. If so, it is critical to understand the underlying causes and mechanisms, which allow these beetles to widen their window of opportunity for suitable hosts.

Adaptations that could facilitate attack by ambrosia beetles on living trees

Wood-boring scolytid beetles secure their host habitat by either killing the entire host plant, killing only part of a larger host, or breeding in dying or dead tissue (Kirkendall et al., 1997). The later option is the easiest, and in evolutionarily terms probably the most basic strategy of host colonization, because it does not involve a special capacity to overcome constitutive or induced host resistance. Beetles that follow this strategy are opportunists, that take advantage of evanescent hosts (Atkins, 1966b). Invading and breeding in living tissue is a much more demanding task that requires an ability to overcome or avoid a tree's defenses. Bark beetles that invade vigorous healthy tress employ two tactics, mass attacking a tree in large numbers, and inoculating the tree with pathogenic fungi that suppress the tree's capacity to activate resistance mechanisms (Kirkendall et al., 1997; Raffa, 1991). A few bark beetles have evolved the ability to neutralize or endure local defense mechanisms in single-family groups, e.g. *Dendroctonus micans* (Kugelann) (Grégoire, 1988). On close examination, some ambrosia beetles also exhibit specialized adaptations, which enable them to attack and reproduce in living trees. Climatic warming (Hepting, 1963) and increased movement of insects through international trade (Krcmar-Nozic et al., 2000) may alter the insect-

fungus-host interaction, which in turn may increase the beetles' ability to attack living trees in the future.

The question arises as to how ambrosia beetles may have become capable of attacking trees that seem to have their full capacity for resistance. If either the beetle or the host is introduced, the non-native organism is subject to novel or episodic selection, in which sudden exposure to new biotic and abiotic factors may provide for rapid evolution. A classic example for rapid evolution of pathogenic fungi is the Dutch Elm disease fungus, *Ophiostoma ulmi* (Buism.) Nannf. The non-aggressive strain of this fungus was introduced into the USA in imported elm veneer logs from central Europe and was first reported in Cleveland, Ohio in 1930 (Stipes and Campana, 1981). In North America an aggressive strain of the pathogen evolved (Manion, 1981), which was subsequently transported to England in logs of rock elm for building boats; the North American strain became established and caused extensive mortality of European elms, not only in England, but throughout western Europe (Brasier and Gibbs, 1973). However, this still leaves the question of why native ambrosia beetles become more aggressive and why they may be successful in attacking living trees? In the following section, I describe adaptations that I hypothesize may facilitate attack by ambrosia beetles on living trees in a changing environment.

Early flight

In temperate climates some ambrosia beetles, e.g. *Gnathotrichus* spp. (Prebble and Graham, 1957), overwinter in their host, usually as larvae that require warm spring temperatures to mature. Others, e.g. *T. lineatum* (Dyer and Kinghorn, 1961), overwinter as emerged adults at the base of trees and stumps and in litter and duff on the forest floor.

Although they may be in reproductive diapause (Fockler and Borden, 1973), they remain mobile and in early spring they can position themselves where temperatures are favorable.

On the B.C. coast, *T. lineatum*, which is almost never found in living, healthy conifers, characteristically does not fly before ambient temperatures reach 15.6 °C (Chapman and Kinghorn, 1958), usually in March or April. However *Trypodendron* spp. in the B.C. interior first fly at lower temperatures very early in the spring, which occurs later then on the coast. Flight by *T. retusum* in northern B.C. began in mid April and peaked at the end of April 1999 (Lindgren et al., 2000); *T. rufitarsus*, *T. retusum* and *T. betulae* in southern B.C. were captured in pheromone baited traps, when daily maximum temperatures reached 8°C (I. M. Wilson, PheroTech Inc., pers. comm.). In aspen forests of southern B.C. I captured *T. retusum* >4 weeks before budflush, and *T. rufitarsus* flew when the ground was still covered with snow. On the coast I captured the exotic *T. betulae* and *T. domesticum* in lineatin-baited traps as early as February, about 4 weeks before budflush by angiosperm trees.

I hypothesize that early flight, prior to budflush, would allow angiosperm-infesting ambrosia beetles to attack their hosts long before the trees begin to photosynthesize. Prior to budflush their metabolic capabilities to withstand attack through induced resistance would be at best weak. Because budflush is at least partially regulated by photoperiod (Fowells, 1965) and the post-diapause activity of beetles is thermally-regulated, the temporal window of opportunity for ambrosia beetles to attack living host trees successfully, may expand in the early spring if climatic warming is occurring. The

beetles could then penetrate the bark, start a gallery system, and establish fungal cultures while the tree is still dormant and unable to resist.

Alternatively it is conceptually possible that vulnerability to attack is heightened by altered winter and spring temperatures caused by climatic warming, in which cold requirements for vernalization are not met, and dormancy persists beyond the normal photophase threshold (Brown, 2002). This would be accompanied by long period of anaerobic metabolism. The window of opportunity for beetles to attack living trees would thus be temporally enlarged in part by ethanol build up, while the capacity for resistance by the living tree would remain low. Under normal winter and spring conditions with prolonged low temperatures that satisfy the criteria for vernalization, the period of anaerobic metabolism would be shorter, and there would be little accumulation of ethanol which signal a vulnerable host.

Nutritional Independence

All scolytid bark beetles must feed on phloem tissue of their host trees, usually as attacking adults and early instar larvae, and often throughout larval development (Stark, 1982). Thus the nutritious phloem is an essential food source, even when late instar larvae in many species, e.g. the European spruce beetle, *Ips typographus* (L.), and the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, may feed exclusively on microorganisms growing on the wall of chambers constructed by the larvae.

In contrast, ambrosia beetle larvae of all ages feed exclusively on ambrosia fungi that the adult females inoculate into the new galleries (Beaver, 1989). Because the heartwood of conifers is composed of dead cells, containing a large amount of phenolic extractives, it is apparently not a suitable substrate for ambrosia fungi. Thus the host

habitat of conifer-infesting ambrosia beetles is usually limited to the living sapwood. In healthy conifers the sapwood may contain toxic constitutive resin in resin canals that may be severed by attacking beetles, or the sapwood may even produce an even more toxic traumatic resin in response to insect or fungal invasion (Raffa, 1991). Therefore, conifer-infesting ambrosia beetles, e.g. *T. lineatum* and *Gnathotrichus* spp., avoid host resistance by infesting the sapwood of very weak hosts, i.e. logs, stumps and dying trees.

Angiosperm trees are the predominant hosts among ambrosia beetles that attack living trees (Table 1). Unlike the sapwood of conifers, the sapwood of angiosperms lacks extensive oleoresin-based constitutive and induced resistance mechanisms, although other resistance mechanisms, e.g. the production of phenolics, may exist. In addition the heartwood of many angiosperm trees is indistinct, and may not be nearly as toxic a habitat as conifer heartwood. Thus, ambrosia beetles that are not nutritionally dependent on living phloem or sapwood may bypass potential host resistance by penetrating deep into the dead heartwood of a living, healthy tree. Deep galleries are typical of such species as *Notoplatypus elongatus* Lea (Platypodidae), *Megaplatypus mutatus* [= *Platypus sulcatus*], and *Corthylus columbianus*.

In B.C. I have observed galleries of *T. retusum*, in trembling aspen, and *T. betulae* and *T. domesticum* in paper birch, penetrating deep into the heartwood, almost reaching the pith. Even *Gnathotrichus retusus* LeConte, which normally only infests the sapwood of conifers, was found successfully attacking and producing brood in red alder, with galleries penetrating 17.5 cm deep, well into the heartwood (Kühnholz et al., 2000). To reach the heartwood of a living tree, ambrosia beetles must breech the potentially resistant living bark and sapwood. *Notoplatypus elongatus* accomplishes this task by

entering through dead tissue associated with scars on the bole (Kirkendall et al., 1997). Other breeching mechanisms could include early spring attack, when the tree is still dormant, or rapid penetration before induced resistance can be mounted. Water stress or disease decreases a trees' capacity to resist attack, and both factors could be exacerbated by climate change (Hepting, 1963).

The fact that ambrosia beetles feed exclusively on ambrosia fungi and are not directly dependent on the host for nutrition is often given as a reason why these beetles are host generalists. However, this assumption cannot be accepted, until molecular phylogenetic analyses lead to hypotheses about the evolution of the beetle-fungus interaction of generalist and specialist fungus and beetles species, and host-specificity studies can determine the relationship among hosts, beetles and fungi. The adaptive radiation of bark and ambrosia beetles is positively correlated with the diversification of symbiotic *Ophiostoma* spp. (Farrell et al., 2001), suggesting a trend towards species specificity of fungal hosts.

In addition, I have found that all *Trypodendron* spp. in B.C. have their own specific mycobiota, mostly *Ophiostoma* spp., and some *Trypodendron* spp. are highly host specific. One type of fungus is always associated with the larval cradles, while another is found principally on the main gallery walls and may also penetrate the adjacent wood. This suggests that one fungus has its primary role as larval food, while another might facilitate the living space in the host tissue. The highly specialized mycangia, in which spores of the associated fungi overwinter and are transported to new hosts (Malloch and Blackwell, 1993), may also ensure highly specific beetle-fungal associations. Thus, symbiotic fungi allow the beetle to be nutritionally independent from

their host trees, but nutritional independence may not be enhanced by the ability of the fungus to grow in multiple host species.

Potentially Pathogenic Fungi

Attacks by beetles in the families Scolytidae and Platypodinae are often mediated by beetle-vectored symbiotic fungi from the genera *Ophiostoma* and *Ceratocystis* and their anamorph (class Ascomycetes), commonly refered to as "ophiostomatoids" (Wingfield et al., 1993). For example *O. clavigerum* is associated with *D. ponderosae* (Bramble and Holst, 1940; Paine et al., 1997), and *O. ulmi*, causes Dutch Elm disease in association with *Scolytus* spp. (Brasier and Gibbs, 1973). International transportation of beetles may have caused rapid evolution of introduced plant pathogens via hybridization (Brasier, 2001), producing even more aggressive virulent new strains than their parent strains or species.

The potential for pathogenicity in ambrosia fungi has yet to be investigated. However, the fungi living in the gallery of ambrosia beetles penetrate the often living sapwood, as evidenced by the dark coloration ("stain") of the pigmented hyphae. My observations of patches of exposed dead wood containing *T. retusum* galleries in otherwise healthy trembling aspens suggest that mutualistic *Ophiostoma* spp. are sufficiently pathogenic to kill host tissue within centimetres of the beetles' tunnels.

These observations lead us to several hypotheses not yet tested. One is that under changing climatic conditions that alter attack dynamics in increasingly stressed hosts, normally non-pathogenic ambrosia fungi may become pathogenic. Stress may also be caused by pathogenic fungi, e.g. *Phytophthora ramorum* Werres, A.W.A.M. de Cock (Pythiaceae) on oaks in California that predisposes its hosts to mass attack by

Monarthrum scutellare LeConte (McPherson et al., in press). A second hypothesis is that fungi associated with exotic ambrosia beetles may express increased pathogenicity in new hosts with which they have not co-evolved. A third and most alarming hypotheses is that as for *O. ulmi* (Brasier, 2001), hybridization may have occurred, or could occur, between species or strains of ambrosia fungi to produce a new strain with heightened pathogenicity.

In B.C., it is only because of the diligence of L. M. Humble that we know of the five species of exotic ambrosia beetles that are established (Humble, 2001). It is highly possible that in B.C. and elsewhere there have been undetected introductions of exotic beetles, and even exotic geographic strains of indigenous beetles species, e.g. *T. lineatum*, with accompanying hybridization of fungi. The latter would be least likely to be detected, because genetic analyses have not been done. Because conifer-infesting ambrosia beetles, e.g. *T. lineatum* and *G. retusus,* occasionally make the mistake of attacking an angiosperm tree (Kühnholz et al., 2000; Lindgren, 1986), there is even the possibility of hybridization among fungi associated with conifer- and angiosperm-infesting ambrosia beetles.

Cryptic Behavior

The wood-boring habit of ambrosia beetles likely facilitated the diversification of beetles/fungus associations (Farrell et al., 2001), as ambrosia beetles exploit the abundant biomass represented by the wood of both gymnosperm and angiosperm trees. In prehistoric times, ambrosia beetles must have occasionally moved considerable distances when infested trees were swept down rivers in spring floods, or carried in oceanic currents. In modern times their cryptic habit promotes concealed and protected

transportation, which may cover a great distance when movement occurs internationally in infested dunnage or in other wood products. Such movement in turn provides the opportunity for hybridization and rapid evolution through introduction of exotic insect, and fungal species and genotypes. Although bark beetles also move in this manner, the bark is often removed in manufacturing or is sloughed off, while ambrosia beetles may remain undisturbed in their wood habitat, and may even complete their life cycles in unseasoned sawn lumber (McLean and Borden, 1975).

Marchant and Borden (1976) noted 18 species of ambrosia beetles that had become established as exotics worldwide. Because exotics have not co-evolved with their new hosts, they may have considerable potential to become more aggressive than on naturally resistant hosts in their native range. Examples of wood-boring insects that have become tree killers as exotics include the wood wasp, *Sirex noctilio* (F.) (Hymenoptera: Siricidae), on radiata pine, *Pinus radiata* D. Don, in New Zealand and Australia (Taylor, 1981), and very recently the brown spruce longhorn beetle, *Tetropium fuscum* (F.) (Coleoptera: Cerambycidae), on red spruce, *Picea rubens* Sarg., in Nova Scotia (Smith and Humble, 2000). It appears that among exotic ambrosia beetles, both *X. dispar* and *X. crassiusculus* have also acquired the ability to kill trees (Table 1). Tree killing potential could be facilitated in a new habitat through a number of processes including early flight that allows insects to attack hosts before the metabolic processess required for induced resistance are activated, by an insect-produced toxin, as occurs for *S. noctilio* (Coutts, 1969), or by normally benign mutualistic fungi expressing pathogenicity in hosts that have not evolved resistance to them, e.g. as occurs for white pine blister rust, *Cronartium ribicoloa* J.C. Fisher ex Rabh., on soft pines in North America (Ziller, 1974).

Complex Chemical Ecology

Ethanol is the key kairomone in the primary attraction of *T. lineatum* and *Gnathotrichus sulcatus* (LeConte) to host conifer logs undergoing anaerobic metabolism under hypoxic conditions (Cade et al., 1970; Graham, 1968; Moeck, 1970). Since these pioneering studies, many other species have been found to orient positively to ethanol. Increasingly convincing evidence indicates that ethanol may be emitted by living trees under some type of stress that will result in anaerobic metabolism, including flooding (Crawfort and Finegan, 1989; Joseph and Kelsey, 1997) and infection by root and stem rots (Gara et al., 1993; Kelsey and Joseph, 1998; Kelsey et al., 1998). In both incidences, ethanol could be used by ambrosia beetles as a signal of stressed and potentially susceptible hosts.

In the spring, trees may come under stress simply by resuming growth before the crown is fully functional, and before soil conditions allow oxygenated water to be drawn up by the roots. The resulting anaerobic metabolism (Eklund, 1990; Harry and Kimmerer, 1991) may cause ethanol concentrations to rise in both the phloem and the xylem sap (Kimmerer and Stringer, 1988; MacDonald and Kimmerer, 1993). This may signal to host-seeking ambrosia beetles that such a tree is a potential host because it is undergoing transitory stress. With a warming climate, anaerobic metabolism in the hypoxic bole of healthy trees may occur increasingly earlier and persist for longer durations than in the past, and may possibly occur in trees that have not entered complete overwintering dormancy (J.C.Gregoire, pers. comm.). Thus, the likelihood of attack by ambrosia beetles in the early spring may be greatly increased.

While positive responses to ethanol may be ubiquitous among host-seeking ambrosia beetles, host specificity and reproductive isolation may also be maintained by responses to semiochemicals. (Klimetzek et al., 1981)) found that European *Trypodendron* spp. all use lineatin as an aggregation pheromone. However, angiosperm-infesting species were repelled by α-pinene from coniferous hosts, and the conifer-infesting *T. lineatum* did not attack lineatin-baited angiosperm logs, because of some unknown chemical deterrent. Similarly, I have found that (+)-lineatin is shared as a pheromone in B.C. by conifer-infesting *T. lineatum* and *T. rufitarsus* and angiosperm infesting *T. retusum*. Host-specificity is apparently maintained by *T. retusum* being repelled by α-pinene from conifers, and the conifer species being repelled by salicylaldehyde from trembling aspen. In turn, the non-host repellents may act with lineatin as synergists in directing each species to its correct host. Thus reproductive isolation is not based solely on pheromones, but requires host and non-host volatiles in synergy with pheromones.

From the above considerations one can assume that similarly efficient mechanisms of olfactory recognition of, and orientation to, stressed hosts of the right species, as well as potential mates and proven hosts already under attack, must occur among all or most species of ambrosia beetles. Thus, they have the necessary tools to locate and colonize increasingly stressed hosts in a changing environment. If this capability is coupled with heightened pathogenicity of mututalistic fungi, and possibly exotic species or new strains vectored by native or exotic beetles, there may be considerable potential for infesting living trees in the future.

Actions

I propose four avenues for research that may lead to an understanding of the threat of ambrosia beetles increasingly attacking living trees in the future, and may also influence the implementation of pest management strategies and tactics.

Surveys

Worldwide surveys of beetles and associated fungi should be conducted at or around international ports of entry with ethanol baited traps as used by (Humble, 2001) in B.C. These may disclose new records of exotic species that could potentially attack living trees. In addition, they may justify continued or strengthened regulatory measures to prevent new introductions. According to the observed early flight of ambrosia beetles and the hypothesized effect of climatic warming, survey trapping should begin very early in the spring, as should any mass-trapping program that is implemented in an area that experiences attacks by exotic species of ambrosia beetles, or altered beetle attack dynamics.

Molecular Genetics

Diagnoses of exotic plants and animals are commonly based on traditional morphological identification. However, identifications of many microorganisms, including new hybrid strains, require molecular markers, DNA sequencing and fingerprinting.

To determine the geographic origins of exotic beetles and ambrosia fungi, and maybe phoretic mites, studies of the molecular genetics of both native and introduced populations are required. They may pinpoint routes of transport or entry that could be

blocked in the future, and may reveal biological similarities between native and introduced populations that could be exploited in their management. In addition, local populations of beetles and fungi that have wide geographic ranges should be examined for the presence of exotic genes that could disclose the occurrence and source of previously undetected introductions, hybridization, and potentially increased virulence.

Pathogenicity

The emergence of *Phytophthora ramorum* as the cause of "Sudden Oak Death", a new disease on oaks (McPherson et al., 2001) is cause for alarm. The possibility that interspecific hybridization among *Phytophthora* spp. (Brasier, 2001) or that conditions necessary for other species e.g. *P. cinnamomi* Rands (Pythiaceae), to express pathogenicity (Roth and Kuhlman, 1966) may be met in a warmer climate (Hepting, 1963) is cause for even greater alarm. Therefore, intensive surveys, including molecular based protocols, for the occurrence of new or potentially virulent pathogens that could predispose living trees to attack by ambrosia beetles are advisable. For example, infections by *Phytophthora* spp. may predispose *Fagus sylvatica* in Belgium to attack by *T. domesticum* and *T. signatum*. These investigations could be coupled with studies to determine the pathogenicity of new fungal isolates from the galleries of exotic ambrosia beetles, or populations determined by molecular analysis to harbour exotic genes.

Chemical Ecology

The refinement of coupled gas-chromatographic-electroantennographic detection analysis (GC-EAD) for bark and ambrosia beetles (Gries, 1995) has made it possible to identify new pheromones and other semiochemicals with little starting material and very

few beetles. I have used GC-EAD to identify a new pheromone for *T. betulae*, as well as numerous potential kairomones used in the detection of hosts and non-hosts by *Trypodendron* spp. The opportunity now exists to discover new semiochemicals for all ecologically or environmentally important species of ambrosia beetles. Tactics for semiochemical-based mass trapping (Lindgren and Fraser, 1994b; McLean and Borden, 1979) or attack prevention (Borden et al., in press) developed for log-infesting ambrosia beetles could then be adapted for use against species that attack living trees.

Conclusion

My discussion of adaptations that may facilitate attack on living trees suggests that it is most probably a combination of more than one factor that makes novel attacks possible. But this phenomenon does not necessarily require changes in host attractiveness or repellency, the trees' capacity to resist attacks, or the virulence of the beetle or ambrosia fungus. A simple change in the timing of the interaction between the beetles and their hosts that has shifted due to a changing climate, could underlie increasing attacks on living trees. Stress by climate change or environmental factors such as air pollution may make hosts more vulnerable to infections by pathogens, which in turn could predispose the host to attack by ambrosia beetles. The two critical question to ask are: 1) why can ambrosia beetles attack apparently healthy trees, and 2) what conditions have changed to widen the window of opportunity for beetles and their fungal symbionts to attack living trees? Further knowledge is required to facilitate a proactive and rapid response to the risk of introductions of exotic insects, their symbiotic fungi and associated mites, and to ameliorate the conditions that could weaken the resistance of living trees to attack by ambrosia beetles.

LITERATURE CITED

Abrahamson, L.P., H.-M. Chu, and D.M. Norris. 1967. Symbiotic interrelationships between microbes and ambrosia beetles. II. The organ of mycrobial transport and perpetuation in *Trypodendron betulae* and *T. retusum* (Coleoptera: Scolytidae). *Annals of the Entomological Society of America* 60:1107-1110.

Abrahamson, L.P., and D.M. Norris. 1966. Symbiotic interrelationships between microbes and ambrosia beetles. II. The organ of mycrobial transport and perpetuation in *Xyloterinus politus*. *Annals of the Entomological Society of America* 59:877-880.

Agrios, G.N. 1988. Plant Pathology. Academic Press, San Diego, California.

Anderson, D.M. 1974. First record of *Xyleborus semiopacus* in the continental United States (Coleoptera: Scolytidae). *U.S. Department of Agriculture Cooperative Economic Insect Report* 24:863-864.

Andersson, M. 1994. Sexual Selection. Princeton University Press, Princeton, New Jersey.

Arn, H., E. Städler, and S. Rauscher. 1975. The electroantennographic detector-A selective and sensitive tool in the gas chromatographic analysis of insect pheromones. *Zeitschrift für Naturforschung* 33c:722-725.

Atkins, M.D. 1966a. Behavioral variation among Scolytidae in relation to their habitat. *The Canadian Entomologist* 98:285-288.

Atkinson, T.H. 1988. Effects of climate and vegetation on patterns of host use by bark beetles and ambrosia beetles (Coleoptera: Scolytidae and Platypodidae). *Environmental Entomology*.

Ayres, B.D., M.P. Ayres, M.D. Abrahamson, and S.A. Teale. 2001. Resource partitioning and overlap in three sympatric species of *Ips* bark beetles (Coleoptera: Scolytidae). *Oecologia* 128:443-453.

Ayres, M.P., R.T. Wilkens, J.J. Ruel, M.J. Lombardero, and E. Vallery. 2000. Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi. *Ecology* 81:2198-2210.

Baker, J.M. 1963. Ambrosia beetles and their fungi, with particular reference to *Platypus cylindrus* Fab. *Symposia Society of General Microbiology* 13:231-265.

Baker, T.C. 2002. Mechanism of saltational shifts in pheromone communication systems. *Proceedings of the National Academy of Science, USA* 99:13368-13370.

Barras, S.J., and T. Perry. 1971. Gland cells and fungal associated with prothoracic mycangium of *Dendroctonus adjunctus* (Coleoptera: Scolytidae). *Annals of the Entomological Society of America* 64:123-126.

Barras, S.J., and T. Perry. 1972. Fungal symbionts in the prothoracic mycangium of *Dendroctonus frontalis* (Coleopt.: Scolytidae). *Zeitschrift für angewandte Entomologie* 71:95-104.

Batra, L.R. 1963. Ecology of ambrosia fungi and their dissemination by beetles. *Transactions of the Kansas Academy of Science* 66:213-236.

Batra, L.R. 1966. Ambrosia fungi: Extent of specificity to ambrosia beetles. *Science* 153:193-195.

Batra, L.R. 1967. Ambrosia fungi: A taxonomic revision, and nutritional studies of some species. *Mycology* 59:976-1017.

Batra, L.R. 1972. Ecosymbiosis between ambrosi fungi and beetles-I. *Indian Journal of Mycology and Plant Pathology* 2:165-169.

Batra, L.R., and S.W.T. Batra. 1979. Termite-fungus mutualism. *In* Insect-Fungus Symbiosis: Nutrition, Mutualism and Commensalism. L.R. Batra, editor. Wiley, New York.

Batra, L.R., S.W.T. Batra, and T. Nakashima. 1986. Some techniques to study ambrosia beetles and their associated fungi. *Memoirs of Hokkaido Musashi Women's Junior College* 18:74-94.

Batra, L.R., and M. Downing Michie. 1963. Pleomorphism in some ambrosia and related fungi. *Transactions of the Kansas Academy of Science Mycolological Society* 66:470-481.

Batra, S.W.T., and L.R. Batra. 1967. The fungus gardens of insects. *Scientific American* 217:112-120.

Bauer, J., and J.P. Vité. 1975. Host selection by *Trypodendron lineatum*. *Naturwissenchaften* 62:539.

Beaver, R.A. 1989. Insect-fungus relationships in bark and ambrosia beetles. *In* Insect-Fungus Interactions. N.M. Wilding, N.M. Collins, P.M. Hammond, and J.F. Webber, editors. Academic Press, London, pp.121-143.

Bhagwandin, H.O. 1993. The shothole borer: an ambrosia beetle of concern for chestnut orcharding in the Pacific Northwest. *Northern Nut Growers Association Annual Report* 84:168-177.

Birch, M.C. 1974. Pheromones. North-Holland Publ. Co., New York.

Birch, M.C. 1984. Aggregation in bark beetles. *In* Chemical Ecology of Insects. W.J. Bell and R.T. Cardé, editors. Chapman and Hall, New York, pp. 331-353.

Birch, M.C., D.M. Light, D.L. Wood, L.E. Browne, R.M. Silverstein, B.J. Bergot, G. Ohloff, J.R. West, and J.C. Young. 1980. Pheromonal attraction and allomonal interruption of *Ips pini* in California by the two enantiomers of ipsdienol. *Journal of Chemical Ecology* 6:703-717.

Birgersson, G., G.L. Debarr, P. De Groot, M.J. Dalusky, J. Pierce H. D., J.H. Borden, J.H. Meyer, W. Francke, K.E. Espelie, and C.W. Berisford. 1995. Pheromones in the white pine cone beetle, *Conophthorus coniperda* (Schwarz) (Coleoptera: Scolytidae). *Journal of Chemical Ecology* 21:143-167.

Borden, J.H. 1977. Behavioral responses of Coleoptera to pheromones, allomones, and kairomones. *In* Chemical Control of Insect Behavior: Theory and Application. H.H. Shorey and J.J. McKelvey, editors. Wiley, New York.

Borden, J.H. 1982a. Aggregation pheromones. *In* Bark Beetles in North American Conifers. J.B. Mitton and K.B. Sturgeon, editors. University of Texas Press, Austin, Texas.

Borden, J.H. 1982b. Secondary attraction in the Scolytidae: an annotated bibliography. *Simon Fraser University Pest Management Paper No. 26.*

Borden, J.H. 1984. Semiochemical-mediated aggregation and dispersion in Coleoptera. *In* Insect Communication. W.T. Lewis, editor. Academic, New York.

Borden, J.H. 1985. Aggregation pheromones. *In* Comprehensive Insect Physiology, Biochemistry and Pharmacology. Vol. 9. G.A. Kirkut and L.I. Gilbert, editors. Pergamon Press, Oxfort.

Borden, J.H. 1988. The striped ambrosia beetle. *In* Dynamics of Forest Insect Populations. A.A. Berryman, editor. Plenum, New York.

Borden, J.H. 1993. Strategies and tactics for the use of semiochemicals against forest insect pests in North America. *In* Pest Management: Biologically Based Technologies. R.D. Lumsden and J.L. Vaughn, editors. American Chemical Society, Washington, DC.

Borden, J.H. 1997. Disruption of semiochemical-mediated aggregation in bark beetles. *In* Insect Pheromone Research: New Directions. R.T. Cardé and A.K. Minks, editors. Chapman and Hall, New York.

Borden, J.H., L.J. Chong, R. Gries, and H.D. Pierce, Jr. 2001. Nonhost volatiles as repellents for the striped ambrosia beetle. *Integrated Pest Management Reviews* 3/4.

Borden, J.H., L.J. Chong, K.N. Slessor, A.C. Oehlschlager, H.D. Pierce, Jr., and B.S. Lindgren. 1981. Allechemie activity of aggregation pheromone between three sympatric species of ambrosia beetles (Coleoptera: Scolytidae). *The Canadian Entomologist* 113:557-563.

Borden, J.H., J.R. Handley, B.D. Johnston, J.A. MacConnell, R.M. Silverstein, K.N. Slessor, A.A. Swigor, and W.T. W. 1979. Synthesis and field testing of 4,6,6-lineatin, the aggregation pheromone of *Trypodendron lineatum* (Coleoptera: Scolytidae). *Journal of Chemical Ecology* 5:681-689.

Borden, J.H., J.R. Handley, J.A. McLean, R.M. Silverstein, L. Chong, K.N. Slessor, B.D. Johnston, and H.R. Schuler. 1980a. Enantiomer-based specificity in pheromone communication by two sympatric *Gnathotrichus* species (Coleoptera: Scolytidae). *Journal of Chemical Ecology* 6:445-456.

Borden, J.H., D.W.A. Hunt, D.R. Miller, and K.N. Slessor. 1986. Orientation in forest Coleoptera: an uncertain outcome of responses by individual insects to variable stimuli. *In* Mechanisms in Insect Olfaction. T.L. Payne, M.C. Birch, and C.E.S. Kennedy, editors. Clarendon Press, Oxford.

Borden, J.H., C.J. King, B.S. Lindgren, L. Chong, D.R. Gray, A.C. Oehlschlager, K.N. Slessor, and H.D. Pierce, Jr. 1982. Variation in the response of *Trypodendron lineatum* from two continents to semiochemicals and trap form. *Environmental Entomology* 11:403-408.

Borden, J.H., A.C. Oehlschlager, K.N. Slessor, L. Chong, and J. Pierce H.D. 1980b. Field tests of isomeres of lineatin, the aggregation pheromone of *Trypodendron lineatum* (Coleoptera: Scolytidae). *Canadian Entomologist* 112:107-109.

Borden, J.H., and C.E. Slater. 1969. Sex pheromone of *Trypodendron lineatum*: Production in the female hindgut-malpighian tubule region. *Annals of the Entomological Society of America* 62:454-455.

Borden, J.H., I.M. Wilson, R. Gries, L.J. Chong, H.D. Pierce, Jr., and G. Gries. 1998. Volatiles from the bark of trembling aspen, *Populus tremuloides* Michx. (Salicaceae) disrupt secondary attraction by the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). *Chemoecology* 8:69-75.

Borg-Karlson, A.-K., C.R. Unelius, I. Valterova, and L.A. Nilsson. 1996. Floral fragrance chemistry in the early flowering shrub *Daphna mezereum*. *Phytochemistry* 41:1477-1483.

Boughman, J.W. 2001. Divergent sexual selection enhances reproductive isolation in stickelbacks. *Nature* 411:944-948.

Bramble, W.C., and E.C. Holst. 1940. Fungi associated with *Dendroctonus frontalis* in killing shortleaf pines and their effects on conduction. *Phytopathology* 30:881-899.

Brand, J.M., J.W. Bracke, L.N. Britton, A.J. Markovetz, and S.J. Barras. 1976. Bark beetle pheromones: Production of verbenone by mycangial fungus of *Dendroctonus frontalis*. *Journal of Chemical Ecology* 2:195-199.

Brasier, C.M. 2001. Rapid evolution of introduced plant pathogens via interspecific hybridazation. *BioScience* 51:123-133.

Brasier, C.M., and J.N. Gibbs. 1973. Origin of the Dutch elm disease epidemic in Britain. *Nature* 241:607-609.

Brasier, C.M., and S.A. Kirk. 1993. Sibling species within *Ophiostoma piceae*. *Mycological Research* 97:811-816.

Bridges, J.R., and J.C. Moser. 1983. Role of phoretic mites in transmission of blue stain fungi. *Ecological Entomology* 8:9-12.

Bright, D.E., and R.E. Skidmore. 2000. A cataloge of Scolytidae and Platypodidae (Coleoptera), Supplement 2 (1995-1999). NRC Research Press, Ontario, Canada. 523 pp.

Bright, D.E.J. 1968. Review of the tribe Xyleborini in America north of Mexico (Coleoptera: Scolytidae). *The Canadian Entomologist* 100:1289-1323.

Bright, D.E.J. 1976. The Insects and Arachnids of Canada Part 2: The Bark Beetles of Canada and Alaska (Coleoptera: Scolytidae). Canada Department of Agriculture, Publication 1576, Ottawa.

Brown, K. 2002. Surviving the long nights: How plants keep their cool. *Science* 297:1267-1268.

Browne, F.G. 1965. Types of ambrosia beetle attack on living trees in the tropical forests. *In* XII th International Congress of Entomology. Vol. 680, London, England.

Butlin, R. 1987. Speciation by reinforcement. *Trends in Ecology and Evolution* 2:8-13.

Butlin, R. 1995. Reinforcement: an idea evolving. *Trends in Ecology & Evolution* 10:432-434.

Byers, J.A. 1989. Chemical ecology of bark beetles. *Experientia* 45:271-283.

Byers, J.A. 1995. Host-tree chemistry affecting colonization in bark beetles. *In* Chemical Ecology of Insects. R.T. Cardé and W.J. Bell, editors. Chapman and Hall, New York.

Byers, J.A., Q.H. Zhang, and G. Schlyter. 1998. Volatiles from nonhost birch trees inhibit pheromone response in spruce bark beetles. *Naturwissenschaften* 85:557-561.

Byrne, K.J., W.E. Gore, G.T. Pearce, and R.M. Siverstein. 1975. Porapak-Q collection of airborne organic compounds serving as models for insect pheromones. *Journal of Chemical Ecology* 1:1-7.

Cade, S.C., B.F. Hrutfiord, and R.I. Gara. 1970. Identification of a primary attractant for *Gnathotrichus sulcatus* isolated from western hemlock logs. *Journal of Economic Entomology* 63:1014-1015.

Cane, J.H., M.W. Stock, D.L. Wood, and S.J. Gast. 1990. Phylogenetic relationship of *Ips* bark beetles (Coleoptera: Scolytidae): electrophoretic and morphometric analysis of the *grandicollis* group. *Biochemical Systematics and Ecology* 18:359-368.

Cardé, A.M., T.C. Baker, and R.T. Cardé. 1979. Identification of a four component sex pheromone of the female oriental fruit moth, *Grapholitha molesta* (Lepidoptera: Tortricidae). *Journal of Chemical Ecology* 5:423-427.

Cardé, R.T. 1986. The role of pheromone in the reproductive isolation and speciation of insects. *In* Evolutionary Genetics of Invertebrate Behavior: Progress and Prospects. H.M. D., editor. Plenum Press, New York and London.

Cardé, R.T., A.M. Cardé, A.S. Hill, and W.L. Roelofs. 1977. Sex pheromone specificity as a reproductive isolation mechanism among the sibling species *Archips argyropilus* and *A. mortunanus* and other sympatric tortricine moths (Lepidoptera: Tortricidae). *Journal of Chemical Ecology* 3:747-752.

Campbell, S.A. (2004) Integration of visual and olfactory cues by host-seeking forest coleoptera (Scolytidae, Cerambycidae). Master thesis, Department of Biological Sciences Burnaby, British Columbia, Simon Fraser University.

Chapin, J.B., and A.D. Oliver. 1986. New record for *Xylosandrus* and *Xyleborus* species (Coleoptera: Scolytidae). *Proceedings of the Entomological Society of Washington* 88:680-683.

Chapman, J.A. 1962. Field studies on attack flight and log selection by the ambrosia beetle *Trypodendron lineatum* (Oliv.) (Coleoptera, Scolytidae). *The Canadian Entomologist* 94:74-92.

Chapman, J.A., and J.M. Kinghorn. 1958. Studies of the flight and attack activity of the ambrosia beetle, *Trypodendron lineatum* (Oliv.), and other scolytids. *The Canadian Entomologist* 90:362-372.

Chikashita, H., K. Hirao, and K. Itoh. 1993. Stereoselective synthesis of *trans* isomers of a volatile compound isolated from elm bark beetle "*Pteleobius vittatus*" via stereospecisfic cyclohdehydration route. *Bulletin of the Chemical Society of Japan* 66:1738-1742.

Cognato, A.I., S.J. Seybold, D.L. Wood, and S.A. Teale. 1997. A cladistic analysis of pheromone evolution in *Ips* bark beetles. *Evolution* 51:313-318.

Costilla, M.A., and M.E. Venditti. 1992. Importancia y control del taladrillo *Platypus sulcatus*. *Revista Industrial y Agricola de Tucuman* 69: 1-2:163-166.

Coutts, M.P. 1969. The mechanism of pathogenicity of *Sixex noctilio* on *Pinus radiata*. II. Effects of *S. noctilio* mucus. *Australian Journal of Biological Sciences* 22:1153-1161.

Coyne, J.A., A.P. Crittenden, and K. Mah. 1994. Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. *Science*. 265:1461-1464.

Coyne, J.A., and H.A. Orr. 1997. 'Patterns of speciation in *Drosophila*' revisited. *Evolution*. 51:295-303.

Coyne, J.F., and L.H. Lott. 1976. Toxicity of substances in pine oleoresin in southern pine beetles. *Journal of Georgian Entomological Society* 11:301-305.

Crawfort, R.M.M., and D.M. Finegan. 1989. Removal of ethanol from lodgepole pine roots. *Tree Physiology* 5:53-61.

Crespi, B.J., and C.P. Sandoval. 2000. Phylogenetic evidence for the evolution of ecological specialization in *Timema* walking-sticks. *Journal of Evolutionary Biology* 13:249-262.

Dallara, P.L., S.J. Seybold, W. Francke, and D.L. Wood. 1994. The chemical ecology of *Pityophthorus* Eichoff (Coleoptera: Scolytidae) in central coastal California. *In* California Forest Pest Council, Proceedings of the 43rd Annual Meeting. Appendix, pp. lxvii-lxix. D.H. Adams, J.E. Rois, and A.J. Storer, editors, Rancho Cordova, California.

Daterman, G.E., J.A. Rudinksy, and W.P. Nagel. 1965. Flight pattern of bark and timber beetles associated with coniferous forests of western Oregon. *Oregon State University Agricultural Experimnetal Station Technical Bulletin No. 87.*

Day, R.W., and G.P. Quinn. 1989. Comparison of treatments after an analysis of variance in ecology. *Ecological Monographs* 59:433-463.

Deglow, E.K., and J.H. Borden. 1998a. Green leaf volatiles disrupt and enhance response to aggregation pheromones by the ambrosia beetle, *Gnathotrichus retusus* (Coleoptera: Scolytidae). *Journal of the Entomological Society of British Columbia* 95:9-15.

Deglow, E.K., and J.H. Borden. 1998b. Green leaf volatiles disrupt and enhance response to aggregation pheromones by the ambrosia beetle, *Gnathotrichus sulcatus* (Coleoptera: Scolytidae). *Canadian Journal of Forest Research* 28:1697-1705.

Delorme, L., and F. Lieutier. 1990. Monoterpene composition of the performed and induced resins of Scots pine, and their effect on bark beetles and associated fungi. *European Journal of Forest Pathology* 20:304-30.

Deyrup, M.A., and T.H. Atkinson. 1987. New distribution records of Scolytidae from Indiana and Florida. *Great Lakes Entomologist* 20:67-68.

Dobzhansky, T. 1940. Genetics and the Origin of Species. Columbia University, New York.

Dowding, P. 1969. The dispersal and survival of spores of fungi causing bluestain in pine. *Transaction of the British Mycological Society* 52:125-137.

Dowding, P. 1984. The evolution of insect-fungus relationships in the primary invasion of forest timber. *In* Invertebrate-Microbial Interactions. J.M. Anderson, A.D.M. Rayner, and D.W.H. Walton, editors. Cambridge University Press, Cambridge, pp. 133-153.

Drès, M., and J. Mallet. 2002. Host races in plant feeding insects and their importance in sympatric speciation. *Philosophical Transactions of the Royal Society of London B* 357:471-492.

Duff, C.M., G. Gries, K. Mori, S. Y., S. M., H. Takikawa, T. Sheng, K.N. Slessor, R. Gries, C.T. Maier, and D.C. Ferguson. 2001. Does pheromone biology of *Lambdina athasaria* and *L. pellucidaria* contribute to their reproductive isolation? *Journal of Chemical Ecology* 27:431-42.

Dyer, E.D.A., and J.M. Kinghorn. 1961. Factors influencing the distribution of overwintering ambrosia beetles, *Trypodendron lineatum* (Oliv.). *The Canadian Entomologist* 93:746-759.

Eklund, L. 1990. Endogenous levels of oxygen, carbon dioxide and ethylene in stems of Norway spruce trees during one growing season. *Trees* 4:150-154.

Elkinton, J.S., and D.L. Wood. 1980. Feeding and boring behavior of the bark beetle *Ips paraconfusus* (Coleoptera: Scolytidae) on the bark of a host and non-host tree species. *The Canadian Entomologist* 112:797-809.

Evenden, M.L., G.J.R. Judd, and J.H. Borden. 1999. A synomone imparting distinct sex pheromone communication channels for *Choristoneura rosaceana* (Harris) and *Pandemis limitata* (Robinson) (Lepidoptera: Tortricidae). *Chemoecology* 9:73-80.

Farrell, B.D. 1998. "Inordinate fondness" explained: Why are there so many beetles? *Science* 281:555-559.

Farrell, B.D., and C. Mitter. 1993. Phylogenetic determinatnce of insect/plant community diversity. *In* Species Diversities in Ecological Communities: Historical and Geographical Perspectives. R.E. Ricklefs and D. Schluter, editors. University of Chicago Press, Chicago.

Farrell, B.D., A.S. Sequeira, B.C. O'Meara, B.B. Normark, C. J.H, and B.H. Jordal. 2001. The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). *Evolution* 55:2011-2027.

Faulds, W. 1973. Discoloration associated with *Platypus* wounds in living *Nothofagus fusca*. *New Zealand Forest Service Report* 3:331-341.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* 17:368-376.

Finnegan, R.J. 1972. Notes on the biology of the pitted ambrosia beetle, *Corthylus punctatissimus* (Coleoptera: Scolytidae) in Ontario and Quebec. *The Canadian Entomologist* 99:49-54.

Fockler, C.E., and J.H. Borden. 1973. Sexual behavior and seasonal mating activity of *Trypodendron lineatum* (Coleoptera: Scolytidae). *The Canadian Entomologist* 104:1841-1853.

Fowells, H.A. 1965. Silvics of Forest Trees of the Unites States. United States Departmant of Agriculture, Forest Service, Agriculture Handbook, No. 271, Washington, D.C.

Francke, W., J. Bartels, H. Meyer, F. Schröder, U. Kohnle, E. Baader, and J.P. Vité. 1995. Semiochemicals from bark beetles (Scolytidae). *Evolution* 52:1731-1743.

Francke, W., G. Hindorf, and W. Reith. 1978. Methyl-1,6-dioxaspiro[4.5]decanes as odors of *Paravespula vulgaris* (L.). *Angewandte Chemie International Ed. in English* 17:862.

Francke-Grosmann, H. 1956. Hautdrüsen als Träger der Pilszymbiose bei Ambrosiakäfern. *Zeitschrift für Morphology und Ökologie der Tiere* 45:275-308.

Francke-Grosmann, H. 1958. Über die Ambrosiazucht holzbrütender Ipiden im Hinblick auf das System. *Verhandlungsbericht der Deutschen Gesellschaft für angewandte Entomologie* 1957:139-144.

Francke-Grosmann, H. 1967. Ectosymbiosis in wood-inhabiting insects. *In* Symbiosis. Vol. 2. S.M. Henry, editor. Academic Press, New York, pp. 141-205.

French, J.R.J., and R.A. Roeper. 1972. Interactions of the ambrosia beetle, *Xyleborus dispar* (Coleoptera: Scolytidae), with its symbiotic fungus *Ambrosiella hartigii* (Fungi imperfecti). *The Canadian Entomologist* 104:1635-1641.

Frumhoff, P.C., and H.K. Reeve. 1994. Using phylogenies to test hypothesis of adaptation: A critique of some current proposals. *Evolution* 48:172-180.

Funk, A. 1965. The symbiotic fungi of certain ambrosia beetles of British Columbia. *Canadian Journal of Botany* 43:929-932.

Furniss, R.L., and V.M. Carolin. 1977. Western Forest Insects. *U.S. Forest Service, Miscellaneos Publication* No 1339, Washington. D. C.

Gara, R.I., W.R. Littke, and D.F. Rhoades. 1993. Emission of ethanol and monoterpenes by fungal infested lodgepole pine trees. *Phytochemistry* 34:987-990.

Gibbs, J.N. 1993. The biology of ophiostomatoid fungi causing sapstain in tress and freshly cut logs. *In Ceratocystis* and *Ophiostoma*, Taxonomy, Ecology and Pathogenicity. M.J. Wingfield, K.A. Seifert, and J.F. Webber, editors. American Phytopathogical Society Press, St. Paul, Minnesota.

Goldhammer, D.S., F.M. Stephen, and T.D. Paine. 1990. The effect of the fungi *Ceratocystis minor* (Hedgecock) Hunt, *Ceratocystis minor* (Hedgecock) Hunt var. *barrasii* Taylor, and SJB 122 on reproduction of southern pine beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae). *The Canadian Entomologist* 122:407-418.

Graham, K. 1967. Fungal-insect mutualism in trees and timber. *Annual Review of Entomology* 12:105-126.

Graham, K. 1968. Anaerobic induction of primary chemical attraction of ambrosia beetles. *Canadian Journal of Zoology* 46:905-908.

Grant, P.R., and B.R. Grant. 1989. Sympatric speciation and Darwin's finches. *In* Speciation and its Consequences. D. Otte and J.A. Endler, editors. Sinauer, Sunderland, MA.

Grégoire, J.-C. 1988. The Greater European Spruce Bark Beetle. *In* Dynamics of Forest Insect Populations. A.A. Berryman, editor. Plenum, New York, pp. 455-478.

Grégoire, J.-C., F. Piel, M. De Proft, and M. Gilbert. 2001 Spatial distribution of ambrosia-beetle catches: a possibly useful knowledge to improve mass-trapping. *Integrated Pest Management Review* 6: 237-242

Gries, G. 1995. Prospects of new semiochemicals and technologies. *In* Applications of Semiochemicals for Management of Bark Beetles Infestations- Proceedings of an Informal Conference. S. Salom and K.R. Hobson, editors. United States Department of Agriculture, Forest Service, General Technical Report INT-GTR-318, Ogden, Utah, pp. 44-47.

Gries, G., R. Gries, and J.H. Borden. 1992. 3,3,7-Trimethyl-1,3,5-cycloheptatriene in the volatiles of female mountain pine beetles, *Dendroctonus ponderosae*. *Naturwissenchaften* 79:27-28.

Gries, G., R. Nolte, and W. Sanders. 1989. Computer simulated host selection in *Ips typographus*. *Entomologia Experimentalis et Applicata* 53:211-217.

Gries, R.L., and M.L. McManus. 1965. The relationship of ambrosia beetles and their microsymbionts to sapwood staining in hardwood hosts. *In* Entomological Society of America, Proceedings North Central Branch. 20:135-136.

Haack, R.A., and F. Slansky. 1987. Nutrional ecology of wood-feeding Coleoptera, Lepidoptera, and Hymenoptera. *In* Nutrional Ecology of Insects, Mites and Spiders. F.J. Slansky and J.G. Rodriguez, editors. Wiley, New York, pp. 449-486.

Haanstad, J.O., and D.M. Norris. 1985. Microbial symbionts of the ambrosia beetle *Xyloterinus politus*. *Microbial Ecology* 11:267-276.

Happ, G.M., C.M. Happ, and S.J. Barras. 1971. Fine structure of the prothoracic mycangium, a chamber of the culture of symbiotic fungi, in the southern pine beetle, *Dendroctonus frontalis*. *Tissue and Cell* 3:295-308.

Harrington, T.C. 1981. Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia*.73:1123-1129.

Harrington, T.C. 1993. Disease of conifers caused by species of *Ophiostoma* and *Leptographium*. *In Ceratocystis* and *Ophiostoma*, Taxonomy, Ecology and Pathogenicity. M.J. Wingfield, K.A. Seifert, and J.F. Webber, editors. APS Press, St. Paul, Minnesota, pp. 161-172.

Harrington, T.C., D. McNew, J. Steimel, D. Hofstra, and R. Farrell. 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycolog.* 93:111-136.

Harry, D.E., and T.W. Kimmerer. 1991. Molecular genetics and physiology of alcohol dehydrogenase in woody plants. *Forest Ecology and Management* 43:251-272.

Haynes, K.F., and M.C. Birch. 1986. Temporal reproductive isolation between two species of the plume moths (Lepidoptera: Pterophoridae). *Annals of the Entomological Society of America* 79:210-215.

Hepting, G.H. 1963. Climate and forest diseases. *Annual Review of Phytopathology* 1:31-50.

Higashi, M., G. Takimoto, and N. Yamamura. 1999. Sympatric speciation by sexual selection. *Nature* 402:523-526.

Hillis, D.M., C. Moritz, and B.K. Mable. 1996. Molecular Systematics. Sinauer Associates, Inc, Sunderland, MA.

Hinds, T.E., and R.W. Davidson. 1972. *Ceratocystis* species associated with the aspen ambrosia beetle. *Mycolog.* 64:405-409.

Hölldobler, B., and E.O. Wilson. 1990. The Ants. Harvard University Press, Cambridge, Massachusetts.

Hoover, S.E.R., B.S. Lindgren, C.I. Keeling, and K.N. Slessor. 2000. Enantiomer preference of T*rypodendron lineatum* and effect of pheromone dose and trap length on response to lineatin-baited traps in interior British Columbia. *Journal of Chemical Ecology* 26:667-677.

Hubbard, H.G. 1897. The ambrosia beetles of the United States. *U.S. Department of Agriculture Bulletin* 7: 1-36.

Huber, D.P.W., and J.H. Borden. 2001. Angiosperm bark volatiles disrupt response of Douglas-fir beetle, *Dendroctonus pseudotsugae*, to attractant-baited traps. *Journal of Chemical Ecology* 27:217-233.

Huber, D.P.W., R. Gries, J.H. Borden, and H.D. Pierce, Jr. 1999. Two pheromones of coniferophagous bark beetles found in the bark of nonhost angiosperms. *Journal of Chemical Ecology* 25:805-816.

Huber, D.P.W., R. Gries, J.H. Borden, and H.D. Pierce, Jr. 2000. A survey of antennal responses by five species of coniferophagous bark beetles (Coleoptera: Scolytidae) to bark volatiles of six species of angiosperm trees. *Chemoecology* 132:103-113.

Humble, L.M. 2001. Invasive bark and wood-boring beetles in British Columbia, Canada. *In* Protection of world forests from insect pests: advances in research. Vol. 11. S. Alfaro, S. Salom, K.S.S. Nair, H. Evans, A. Liebhold, F. Lieutier, M. Wagner, K. Futai, and K. Suzuki, editors. International Union of Forest Research Organization, World Series, Vienna, Austria.

Hunt, D.W.A., and J.H. Borden. 1989. Terpene alcohol pheromone production by D*endroctonus ponderosae* and *Ips paraconfusus* (Coleoptera: Scoytidae) in the absence of readily-culturable microorganisms. *Journal of Chemical Ecology* 15:1433-1463.

Inomata, S. 2000. Identification of sex pheromones of *Anadevidia peponis* and *Macdunnoughia confusa* and field tests of their role in reproductive isolation of closely related Plusiinae moths. *Journal of Chemical Ecology* 26:443-454.

Jacobs, K., and M.J. Wingfield. 2001. *Leptographium* Species: Tree Pathogens, Insect Associates, and Agents of Blue-stain. Academic Press, New York.

Joseph, G., and R.G. Kelsey. 1997. Ethanol synthesis and water relations of flooded *Pseudotsuga menziesii* (Mirb.) France (Douglas-fir) seedlings under controlled condictions. *International Journal of Plant Science* 158:844-850.

Kamata, N., K. Esaki, K. Kato, Y. Igeta, and K. Wada. 2002. Potential impact of global warming on deciduous oak dieback caused by ambrosia fungus *Raffaelea* sp. carried by ambrosia beetle *Platypus quercivorus* (Coleptera: Platypodidae) in Japan. *Bulletin of Entomological Research* 92:119-126.

Karlson, P., and A. Butenandt. 1959. Pheromones (ectohormones) in insects. *Annual Review of Entolmology* 4:39-58.

Kelley, S.T., and B.D. Farrell. 1998. Is specialization a dead end? The phylogeny of host use in *Dendroctonus* bark beetes (Scolytidae). *Evolution* 52:1731-1743.

Kelsey, R.G. 1994. Ethanol synthesis in Douglas-fir logs felled in November, January, and March and its relationship to ambrosia beetle attack. *Canadian Journal of Forest Research* 24:2096-2104.

Kelsey, R.G., and G. Joseph. 1998. Ethanol in Douglas-fir with black-stain root disease (*Leptographium wagneri*). *Canadian Journal of Forest Research* 28:1207-1212.

Kelsey, R.G., and G. Joseph. 1999. Ethanol and ambrosia beetles in Douglas-fir logs exposed or protected from rain. *Journal of Chemical Ecology*.25:2793-2809.

Kelsey, R.G., and G. Joseph. 2003. Ethanol in ponderosa pine as an indicator of physiological injury from fire and its relationship to secondary beetles. *Canadian Journal of Forest Research* 33: 870-884

Kelsey, R.G., G. Joseph, and W.G. Thies. 1998. Sapwood and crown symptoms in ponderosa pine infested with black-stain and anosum root disease. *Forest Ecology and Management* 111:181-191.

Kent, D.S., and J.A. Simpson. 1992. Eusociality in the beetle *Australoplatypus incompertus* (Coleoptera: Platypodidae). *Naturwissenschaften* 9:86-87.

Kim, J.-J., S.H. Kim, S. Lee, and C. Breuil. 2003. Distinguishing *Ophiostoma ips* and *Ophiostoma montium*, two bark beetle-associated sapstain fungi. *(Federation European Mycobiological Societies) Microbiology Letters*. 222:187-192.

Kim, S.H., A. Uzunovic, and C. Breuil. 1999. Rapid detection of *Ophiostoma piceae* and *O. quercus* in stained wood using PCR. *Applied and Environmental Microbiolog.* 65: 287-290.

Kimmerer, T.W., and M.A. Stringer. 1988. Alcohol dehydrogenase and ethanol in the stems of trees. *Plant Physiology* 87:693-697.

Kingsolver, J.G., and D.M. Norris. 1977. The interaction of *Xyleborus ferrugineus* (Coleoptera: Scolytidae) behavior and initial reproduction in realtion to its symbiotic fungi. *Annuals of the Entomological Society of America* 70:1-4.

Kirkendall, L.R., D.S. Kent, and K.F. Raffa. 1997. Interactions among males, females and offspring in bark and ambrosia beetles: the significance of living in tunnels for the evolution of social behavior. *In* The Evolution of Social Behavior in Insects and Arachnids. J.C. Choe and B.J. Crespi, editors. Cambridge University Press, Cambridge, Massachusetts, pp. 181-215.

Klein, E., H. Farnov, and W. Rojahn. 1964. Chemistry of the linalool oxides. *Annual Chemical* 675:73-82.

Klepzig, K.D., J. Flores-Otero, R.W. Hofstetter, and M.P. Ayres. 2004. Effects of available water on growth and competition of southern pine beetle associated fungi. *Mycological Research* 108:183-188.

Klimetzek, D., and W. Francke. 1980. Relationship between the enantiomeric composition of α-pinene in host trees and the production of verbenols in *Ips* species. *Experientia* 36:1343-1345.

Klimetzek, D., J.P. Vité, and E. König. 1981. Über das Verhalten mitteleurpäischer *Trypodendon*-Arten gegen gegenüber natürlichen und synthetischen Lockstoffen. *Allgemeine Forst- und Jagdzeitung* 152, Jg.,4:64-70.

Klimetzek, D., J.P. Vité, and K. Mori. 1980. Zur Wirkung und Formulterung des Populationslockstoffes des Nutzholzborkenkäfers *Trypodendron* (= *Xyloterus*) *lineatum*. *Zeitschrift für angewandte Entomologie* 89:57-63.

Kohnle, U., S. Densborn, P. Kölsch, H. Meyer, and W. Francke. 1992. E-7-methyl-1,6-dioxaspiro[4.5]decane in the chemical communication of European Scolytidae and Nitidulidae (Coleoptera). *Journal of Applied Entomology* 114:187-192.

Kok, L.T., D.M. Norris, and H.-M. Chu. 1970. Sterol metabolism as a basis of mutualistic symbiosis. *Nature* 225:661-662.

Kovach, J., and C.S. Gorsuch. 1985. Survey of ambrosia beetle species infesting South Carolina peach orchards and a taxonomic key for the most common species. *Journal of Agricultural Entomology* 2:238-247.

Krcmar-Nozic, E., B. Wilson, and L. Arthur. 2000. The potential impact of exotic forest pests in North America: a synthesis of research. *Victoria, British Columbia: Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, Information Report*. BC-X-387.

Kühnholz, S., J.H. Borden, and R.L. McIntosh. 2000. The ambrosia beetle, *Gnathotrichus retusus* (Coleoptera: Scolytidae) breeding in red alder, *Alnus rubra* (Betulaceae). *Journal of the Entomological Society of British Columbia* 97:103-104.

Landolt, P.J., and T.W. Phillips. 1997. Host plant influenecs on sex pheromone behavior of phytophagous insects. *Annual Review of Entolmology* 42:371-391.

Lanier, G.N., A. Classon, T. Stewart, J.J. Pistol, and R.M. Silverstein. 1980. *Ips pini*: the basis for interpopulational differences in pheromone biology. *Journal of Chemical Ecology* 6:677-687.

Lanier, G.N., and D.L. Wood. 1975. Specificity of response to pheromones in the genus *Ips* (Coleoptera: Scolytidae), in North America. *Journal of Chemical Ecology* 1:9-23.

Leufven, A. 1991. Role of microorganisms in spruce bark beetles-conifer interactions. *In* Microbial Mediation of Plant-Herbivore Interaction. P. Barbosa, V.A. Krischik, and C.G. Jones, editors. Wiley, New York, pp. 467-483.

Lindgren, B.S. 1983. A multiple-funnel trap for Scolytid beetles (Coleoptera). *The Canadian Entomologist* 115:229-302.

Lindgren, B.S. 1986. *Trypodendrn lineatum* (Olivier) (Coleoptera: Scolytidae) breeding in big leaf maple. *Journal of the Entomological Society of British Columbia* 83:44.

Lindgren, B.S., J.H. Borden, L.J. Chong, L.M. Friskie, and D.B. Orr. 1983. Factors influencing the efficiency of pheromone-baited traps for three species of ambrosia beetles (Coleoptera: Scolytidae). *The Canadian Entomologist* 115:303-313.

Lindgren, B.S., and R.G. Fraser. 1994a. Control of ambrosia beetle damage by mass trapping at a dryland sortimg area in British Columbia. *Forest Chronical* 70:159-163.

Lindgren, B.S., and R.G. Fraser. 1994b. Control of ambrosia beetle damage by mass trapping at a dryland log sorting area in British Columbia. *Forestry Chronicle* 70:159-163.

Lindgren, B.S., S.E.R. Hoover, A.M. MacIsaac, C.I. Keeling, and K.N. Slessor. 2000. Lineatin enantiomer preference, flight periods, and effect of pheromone concentration and trap length on three sympatric species of *Trypodendron* (Coleoptera: Scolytidae). *The Canadian Entomologist* 132:877-887.

Linn, C.E.J., and W.L. Roelofs. 1995. Pheromone communication in moths and its role in the speciation process. *In* Speciation and Recognition Concept: Theory and pallication. D. Lambert, M. and H.G. Spencer, editors. The Johns Hopkins University Press, Baltimore.

Linn, C.J., J.L. Feder, S. Nojima, H.R. Dambroski, S.H. Berlocher, and W.L. Roelofs. 2003. Fruit odor discrimination and sympatric host race formation in *Rhagoletis*. *Proceedings of the National Academy of Science* 100:11490-11493.

Linsley, E.G., and G.F. MacLeod. 1942. Ambrosia beetles attacking deciduous fruit trees in California. *Journal of Economic Entomology* 35:601.

Loftus-Hill, J.J., and M.J. Littlejohn. 1992. Reinforcement and reproductive character displacement in *Gastrophryne carolinensis* and *G. olivacea* (Anura: Microhylidae): a re-examination. *Evolution* 46:896-906.

MacConnell, J.A., J.H. Borden, R.M. Silverstein, and R.M. Stokkink. 1977. Isolation and tentative identification of lineatin, a pheromone from the frass of *Trypodendron lineatum* (Coleoptera: Scolytidae). *Journal of Chemical Ecology*. 3:549-561.

MacDonald, R.C., and T.W. Kimmerer. 1993. Metabolism of transpired ethanol by eastern cottonwood (*Populus deltoides* Bartr.). *Plant Physiology* 102:173-179.

Malloch, D., and M. Blackwell. 1993. Dispersal biology of ophiostomatoid fungi. *In Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. M.J. Wingfield, K.A. Seifert, and J.F. Webber, editors. American Phytopathogical Society Press, St. Paul, Minnesota.

Manion, P.D. 1981. Tree disease concept. Prentice Hall, Engelwood Cliffs New Jersey.

Martikainen, P. 2000. Flight period and ecology of *Trypodendron proximum* (Niijima) (Col., Scolytidae) in Finland. *Journal of Applied Entomology* 124:57-62.

Marvaldi, A.E., A.S. Sequeira, C.W. O'Brien, and B.D. Farrell. 2002. Molecular and morphological phylogenetics of weevils (Coleoptera, Curculionoidea): Do niche shifts accompany diversification? *Systematic Biology* 51:761-785.

Mathiesen-Käärik, A. 1953. Eine Übersicht über die gewöhnliche mit Borkenkäfern associierten Bläuepilze in Schweden. *Meddelanden fran Statens Skogsforskninginstitut* 43:1-25.

Mathiesen-Käärik, A. 1960. Study on the ecology, taxonomy and physiology of Swedish insect-associated blue stain fungi, especially the genus *Ceratocystis*. *Oikos*. 11.

Mayer, E. 1942. Systematic and the origin of species. Columbia University Press, New York.

Maynhard Smith, J. 1966. Sympatric speciation. *American Naturalist* 100:637-650.

McLean, J.A. 1985. Ambrosia bettle: a multimillion dollar degrade problem of sawlogs in coastal British Columbia. *Forestry Chronicle* 61:295-298.

McLean, J.A., and J.H. Borden. 1975. *Gnathotrichus sulcatus* attack and breeding in freshly-sawn lumber. *Journal of Economic Entomology* 68:605-606.

McLean, J.A., and J.H. Borden. 1979. An optimal pheromone-based suppression program for an ambrosia beetle, *Gnathotrichus sulcatus*, in a commercial sawmill. *Journal of Economic Entomology* 72:165-172.

McPherson, B.A., D.L. Wood, A.J. Storer, K.N. Maggi, and R.B. Standiford. 2001. Sudden oak death, a new forest disease in California. *Integrated Pest Management Reviews*. 6:243-246.

Meiffen, M., and M. Belin. 1960. Essai de traitment le scolyte des rameaux du cafeier *Xyleborus morstatti* Hage. *Café, Cacao, The* 4:150-58.

Méou, A., N. Bouanah, A. Archelas, X.M. Zhang, R. Guglielmetti, and R. Furstoss. 1991. Synthesis of all four stereoisomers of enatiomerically pure tetrahydro-2,2,6-trimethyl-6-vinyl-2*H*-pyran-3-ol. *Synthesis* 9:681-682.

Miller, D.R., and J.H. Borden. 1990a. Host volatile (β-phellandrene, plus ipsdienol are involved in both primary and secondary attraction of *Ips pini*. *Journal of Chemical Ecology* 16:2519-2531.

Miller, D.R., and J.H. Borden. 1990b. ß-Phellandrene: kairomone for pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). *Journal of Chemical Ecology* 16:2519-2531.

Miller, D.R., and J.H. Borden. 2000. Dose-dependent and species-specific responses of pine bark beetles (Coleoptera: Scolytidae) to monoterpenes in association with pheromones. *The Canadian Entomologist* 132:183-195.

Miller, J.S., and J.W. Wenzel. 1995. Ecological characters and phylogeny. *Annual Review of Entolmology* 40:389-415.

Moeck, H.A. 1968. Electron microscopic studies of antennal sensillae in the ambrosia beetle *Trypodendron lineatum* (Olivier) (Scolytidae). *Canadian Journal of Zoology* 46: 521-556.

Moeck, H.A. 1970. Ethanol as a primary attractance for the ambrosia beetle, *Trypodendron lineatum* (Coleoptera: Scolytidae). *The Canadian Entomologist* 02:985-995.

Moeck, H.A., D.L. Wood, and K.Q. Lindahl Jr. 1981. Host selection behavior of bark beetles (Coleoptera: Scolytidae) attacking *Pinus ponderosa*, with special emphasis on the western pine beetle, *Dendroctonus brevicomis*. *Journal of Chemical Ecology* 7:49-83.

Mori, K., and P. Puapoomchareon. 1988. Syntheis of all the four stereoisomers of tetrahydro-2,2,6-trimethly-2*H*-pryan-ol, a volatile compound from elm bark beetle *Pteleobius vittatus*. *Annalen der Chemie*:175-177.

Moser, J.C., and J.E. Marcias-Samano. 2000. Tarsonemid mites associates of *Dendroctonus frontalis* (Coelpeotera: Scolytidae): implications of the historical biogeography of *D. frontalis*. *Annals of the Entomological Society of America* 64:72-77.

Moser, J.C., and L.M. Roton. 1971. Mites associated with southern pine beetles in Allen Parish, Louisaina. *The Canadian Entomologist* 103:1775-1798.

Moser, J.C., R.C. Wilkinson, and E.W. Clark. 1974. Mites associated with *Dendroctonus frontalis* Zimmerman (Scolytidae: Coleoptera) in Central America and Mexico. *Turrialba* 24:379-381.

Mueller, U.G., and N. Gerardo. 2002. Fungus-farming insects: multiple origins and diverse evolutionary histories. *Proceedings of the National Acadademy of Science* 99:15247-15249.

Mueller, U.G., S.A. Rehner, and T.R. Schultz. 1998. The evolution of agriculture in ants. *Science* 281:2034-2038.

Nakashima, T., C. Goto, and T. Iizuka. 1987. The primary and auxillary ambrosia fungi isolated from the ambrosia beetles, *Scolytoplatypus shogun* Blandford (Coleoptera: Scolytidae) and *Crossotarsus niponicus* Blandford (Coleoptera: Platypodidae). *Journal of the Faculty of Agriculture Hokkaido University* 63:185-208.

Nakashima, T., and T. Iizuka. 1982. Isolation of some microorganisms associated with five species of ambrosia beetles and two kinds of antibiotics produced by Xv-3 strain in the these isolates. *Journal of the Faculty of Agriculture Hokkaido University* 61:60-72.

Neger, F.W. 1908a. Die Pilzkulturen der Nutzholzborkenkäfer. *Zentralblatt für Bakteriology* 20.

Neger, F.W. 1908b. Die pilzzüchtenden *Bostrychiden*. *Naturwissenschaftliche Zeitschrift für Forst- und Landwirtschaft* 6:274.

Neger, F.W. 1909. Ambrosiapilze. II. Die Ambrosia der Holzbohrkäfer. *Berichte der deutschen botanischen Gesellschaft* 27:372.

Nijholt, W.W., and J. Schönherr. 1976. Chemical response behavior of Scolytidae in West Germany and Western Canada. *Canadian Forestry Service Pacific Forestry Centre Research Report* 32:31-32.

Nord, J.C., and M.L. McManus. 1972. The Columbian timber beetle. *United States Department of Agriculture, Forest Service, Forest Pest Leaflet* No.132.

Nordlund, D.A. 1981. Semiochemicals: a review of the terminology. *In* Semiochemicals: Their Role in Pest Control. D.A. Nordlund, R.L. Jones, and W.J. Lewis, editors. John Wiley & Sons, New York.

Norris, D.M. 1979. The mutualistic fungi of *Xyleborini* beetles. *In* Insect-Fungus Symbiosis: Nutrition, Mutualism and Commensalism. L.R. Batra, editor. Wiley, New York.

Nosil, P. 2002. Transition rates between specialization and generalization in phytophagous insects. *Evolution* 56:1701-1706.

Ober, K.A. 2002. Phylogenetic relationship of the carabid subfamily Harpalinae (Coleoptera) based on molecular sequence data. *Molecular Phylogenetics and Evolution* 24:228-248.

Paine, T.D., and M.C. Birch. 1983. Acquisition and maintenance of mycangial fungi in *Dendroctonus brevicomis* LeConte (Coleoptera: Scolytidae). *Environmental Entomology* 12:1384-1386.

Paine, T.D., K.F. Raffa, and T.C. Harrington. 1997. Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* 42:179-206.

Paine, T.D., F.M. Stephen, and R.G. Cates. 1993. Host defense reactions in response to inoculation with *Ophiostoma* species. *In Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology, and Pathogenicity. M.J. Wingfield, K.A. Seifert, and J.F. Webber, editors. APS Press, St. Paul, Minnesota, pp. 219-224.

Panhuis, T.M., R. Butlin, M. Zuk, and T. Tregenza. 2001. Sexual selection and speciation. *Trends in Ecology & Evolution* 16:364-371.

Perny. 1997. Bemerkenswerte Schadauftreten in Österreich. Forstschutz Aktuell Nr. 22. Vol. 2001. http:///www.fbva.bmlf.gv.at/inst4/fs-aktuell/nr22/04.html, May 2003.

Plettner, E. 2002. Insect pheromone olfaction: New targets for the design of species-selective pest control. *Current Medical Chemistry* 9: 1075-1085.

Pierce, A.M., J.H. Borden, and A.C. Oehlschlager. 1981. Olfactory response to beetle-produced volatiles and host-food attractants by *Oryzaephilus surinamensis* and *O. mercator*. *Canadian Journal of Zoology* 59:1980-1990.

Pierce, H.D., Jr., P. de Groot, J.H. Borden, S. Ramaswamy, and A.C. Oehlschlager. 1995. Pheromones in red pine cone beetle, *Conophthorus resinosae* Hopkins, and its synonym, *C. banksianae* Mc Pherson (Coleoptera: Scolytidae). *Journal of Chemical Ecology* 21-:169-185.

Pitman, G.B. 1969. Pheromone response in pine bark beetles: Influence of host volatiles. *Science* 166:905-906.

Poland, T.M., and J.H. Borden. 1998. Disruption of secondary attraction of the spruce beetle, *Dendroctonus rufipennis*, by pheromones of two sympatric species. *Journal of Chemical Ecology* 24:151–337.

Poland, T.M., J.H. Borden, A.J. Stock, and L.J. Chong. 1998. Green leaf volatiles disrupt responses by the spruce beetle, *Dendroctonus rufipennis*, and the western pine beetle, *Dendroctonus brevicomis* (Coleoptera: Scolytidae) to attractant-baited traps. *Journal of the Entomological Society of British Columbia* 95:17-24.

Pophof, B. 1998. Inhibitors of sensillar esterase reversibly block the responses of moth pheromone receptor cells. *Journal of Comparitive Physiology* 183:153-164.

Pophof, B., T. Gebauer, and G. Ziegelberger. 2000. Decyl-thio-trifluoro propanone, a competitive inhibitor of moth pheromone receptors. *Journal of Comparitive Physiology* 186:315-323.

Posada, D., and K.A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14:817-818.

Posada, D., and K.A. Crandall. 2001. Selecting the best-fit model of nucleotide substitution. *Systematic Biology* 50:580-601.

Prebble, M.L., and K. Graham. 1957. Studies of attack by ambrosia beetles in softwood logs on Vancouver Island, British Columbia. *Forest Science* 3:90-112.

Raffa, K.F. 1991. Induced defense reaction in conifer-bark beetle systems. *In* Phytochemical Induction by Herbivores. D.W. Tallamy and M.J. Raupp, editors. Academic Press, New York, pp. 505-530.

Raffa, K.F. 2001. Mixed messages across multiple trophic levels: the ecology of bark beetle communication systems. *Chemoecology* 11: 49-65.

Raffa, K.F., and A.A. Berryman. 1982. Physiological differences between lodgepole pine resistence and susceptability to the mountain pine beetle and associated microorganisms. *Environmental Entomology* 11:486-492.

Raffa, K.F., T.W. Phillips, and S.M. Salom. 1993. Strategies and mechanisms of host colonization by bark beetles. *In* Interactions among Bark Beetles, Pathogens, and Conifers in North American Forests. T.O. Schowalter and G. Filip, editors. Academic Press Ltd., New York.

Rambaut, A. 1996. Se-Al: Sequence alignment editor. Available at http://evolve.zoo.ox.ac.uk/, August 2004.

Rankin, L.J., and J.H. Borden. 1991. Competitive interactions between the mountain pine beetle and the pine engraver in lodgepole pine. *Canadian Journal of Forest Research* 21:1029–1036.

Reid, R.W. 1961. Moisture change in Lodgepole pine before and after attack by mountain pine beetles. *Forestry Chronicle* 37:368-375.

Reid, R.W., H.S. Whitney, and J.A. Watson. 1967. Reactions of lodgepole pine to attack by *Dendroctonus ponderosae* Hopkins and blue stain fungi. *Canadian Journal of Botany* 45:1115-1126.

Renwick, J.A.A., P.R. Hughes, and I.S. Krull. 1976. Selective production of *cis*- and *trans*-verbenol from (-) and (+)-pinene by a bark beetle. *Science* 191:199-201.

Rivas, L.A. 1964. A reinterpretation of the concept of sympatric and allopatric with proposal of the additional terms syntopic and allotopic. *Systematic Zoology* 13:42-43.

Rodríguez, R.L., L.E. Sullivan, and R.B. Cocroft. 2004. Vibration communication and reproductive isolation in the *Echenopa binotata* species complex of treehoppers (Hemiptera: Membracidae). *Evolution* 58:571-578.

Roelofs, W.L. 1995. The chemistry of sex attraction. *In* Chemical Ecology: The Chemistry of Biotic Interaction. T. Eisner and J. Meinwald, editors. National Academic Press, Washington, DC.

Roelofs, W.L., and R.T. Cardé. 1974. Sex pheromones in the reproductive isolation of lepidopterous species. *In* Pheromones. M.C. Birch, editor. North-Holland Publishing Co, Amsterdam.

Roelofs, W.L., W. Liu, G. Hao, H. Jiao, A.P. Rooney, and J. Linn, C. E. 2002. Evolution of moth sex pheromone via ancestral genes. *Proceedings of the National Academy of Science,* 99:13621-13626.

Roth, L.F., and E.G. Kuhlman. 1966. *Phytophthera cinnamoni*, an unlikely threat to Douglas-fir forestry. *Forest Science* 12:147-159.

Rudinsky, J.A., and G.E. Daterman. 1964. Response of the ambrosia beetle *Trypodendron lineatum* (Oliver) to a female-produced pheromone. *Zeitschrift für angewandte Entomologie* 54:300-303.

Rudinsky, J.A., and R.R. Michael. 1973. Sound production in Scolytidae: Stridulation by female *Dendroctonus* beetles. *Journal of Insect Physiology* 19:689-705.

Ryker, L.C., and J.A. Rudinsky. 1976. Sound production in Scolytidae: Aggressive and mating behavior of the mountain pine beetle. *Annals of the Entomological Society of America* 69:677-680.

Salom, S.M., and J.A. McLean. 1988. Semiochemicals for capturing the ambrosia beetle, *Trypodendron lineatum*, in multiple funnel traps in British Columbia. *Journal of the Entomological Society of British Columbia* 85:34-39.

Sasaerila, Y. 2000. Chemical ecology of oil palm bunch moth, *Tirathiba mundella* (Lepidoptera: Pyralidae) and nettle caterpillars, *Darna bradleyi*, *D. trima*, *Setothosea asigna* and *Setora nitens* (Lepidoptera: Limacodidae). Ph.D thesis, Department of Biological Sciences, Simon Fraser University, Burnanby, B. C.

Sasaerila, Y., Gries, R., Gries, G., Khaskin, G., King, S., Takács, S. and Hardi. 2003. Sex pheromone component of male *Tirthaba mundella* (Lepidoptera: Pyralidae). *Chemoecology* 13: 89-93.

Schedl, K.E. 1947. Die Borkenkäfer Baltischen Bernsteins. *Zentralblatt für das Gesamtgebiet der Entomology* 2:12-45.

Schedl, W. 1964. Biologie des gehöckerten Eichenholzbohrers, *Xyleborus mongraphus* Fab (Scolytidae, Coleoptera). *Zeitschrift für angewandte Entomologie* 53:411-428.

Schmidberger, J. 1836. Naturgeschichte des Apfelborkenkäfers *Apate dispar. Beitrag Obstbaumzeitschrift Naturgeschellschsft Ostbaumschädicher Insekten (Linz)* 4:213-230.

Schneider, I.A., and J.A. Rudinsky. 1969. Mycangial glands and their seasonal changes in *Gnathotrichus retusus* and *G. sulcatus. Annals of the Entomological Society of America* 62:39-43.

Schroeder, L.M. 1992. Olfactory recognition of nonhost aspen and birch by conifer bark beetles *Tomicus piniperda* and *Hylurgops palliatus. Journal of Chemical Ecology* 18:1583-1593.

Schurig, V., R. Weber, D. Klimetzek, U. Kohnle, and K. Mori. 1982. Enantiomeric composition of 'Lineatin' in three sympatric ambrosia beetles. *Naturwissenchaften* 69:602-603.

Seifert, K.A. 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis. In Ceratocystis* and *Ophiostoma*, Taxonomy, Ecology and Pathogenicity. M.J. Wingfield, K.A. Seifert, and J.F. Webber, editors. American Phytopathogical Society Press, St. Paul, Minnesota.

Sequeira, A.S., B.B. Normark, and B.D. Farrell. 2000. Evolutionary assembly of conifer fauna: distinguishing ancient from recent associations in bark beetles. *Proceedings of the Royal Society of London B Biological Sciences* 267:2359-2366.

Seybold, S.J., J. Bohlmann, and K.F. Raffa. 2000. Biosynthesis of coniferophagous bark beetle pheromones and conifer isoprenoids: evolutionary perspective and synthesis. *The Canadian Entomologist* 132:697-753.

Seybold, S.J., T. Ohtsuka, D.L. Wood, and I. Kubo. 1995. Enantiomeric composition of ipsdienol: a chemotaxonomic character of North American populations of *Ips* spp. in the *pini* subgeneric group (Coleoptera: Scolytidae). *Journal of Chemical Ecology* 21:995-1016.

Shigo, A.L. 1965. Organism interactions in decay and discoloration in beech, birch and maple. *United States Department of Agriculture, Forest Service Research Paper NE-43.*

Shore, T.L., and J.A. McLean. 1983. A further evaluation of the interaction between the pheromone and two host kairomones of the ambrosia beetle, *Trypodendron lineatum* (Olivier) and *Gnathotrichus sulcatus* (LeConte). *The Canadian Entomologist* 115:1-5.

Simon, C., F. Frati, A. Beckenbach, B.J. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitrochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* 87:651-701.

Six, D.L., and B.J. Bentz. 2003. Fungi associated with the North American spruce beetle, *Dendroctonus rufipennis*. *Canadian Journal of Forest Research* 33:1815-1820.

Six, D.L., and T.D. Paine. 1997. *Ophiostoma clavigerum* is the mycangial fungus of Jeffery pine beetle, *Dendroctonus jefferyi*. *Mycology* 89:858-866.

Six, D.L., and T.D. Paine. 1999. Phylogenentic comparison of ascomycete mycangial fungi and *Dendroctonus* bark beetles (Coleoptera: Scolytidae). *Systematics* 92:159-166.

Slessor, K.N., A.C. Oehlschlager, A.C. Johnson, Pierce H. D., S.K. Grewal, and L.K.G. Wickremesinghe. 1980. Lineatin: regioselective synth Jr. esis and resolution leading to the ciral pheromone of *Trypodendron lineatum*. *Journal of Organic Chemistry* 45:2290.

Smalley, E.B., K.F. Raffa, R.H. Proctor, and K.D. Klepzig. 1993. Tree responses to infections by species of *Ophiostoma* and *Ceratocystis*. *In Ceratocystis* and *Ophiostoma*, Taxonomy, Ecology and Pathogenicity. M.J. Wingfield, K.A. Seifert, and J.F. Webber, editors. APS Press, St. Paul, Minnesota.

Smith, G.A., and L.M. Humble. 2000. The brown spruce longhorn beetle. *Ottawa, Canada: Natural Resources Canada, Canadian Forest Service, Exotic Forest Pest Advisory* No. 5.

Solheim, H., B. Långström, and C. Hellqvist. 1993. Pathenogenicity of the blue-stain fungi *Leptographium wingfieldii* and *Ophiostoma minus* to Scots pine: Effect of tree pruning and inoculum density. *Canadian Journal of Forest Research* 23:1438-1443.

Stark, R.W. 1982. Generalized ecology and life cycles of bark beetles. *In* Bark Beetles in North American Conifers. J.B. Mitton and K.B. Sturgeon, editors. University of Texas Press, Austin, Texas, pp. 21-45.

Stipes, R.J., and R.S. Campana. 1981. Compendium of Elm Diseases. American Phytopathological Society, St. Paul, Minnesota.

Swofford, D.L. 2000. Phylogenetic Analysis using Parsimony (and other methods). Sinauer Associates, Sunderland, MA.

Symonds, M.R.E., and M.A. Elgar. 2003. The mode of pheromone evolution: evidence from bark beetles. *Proceedings of the Royal Society of London-B. Biological Sciences* Published online: DOI 10. 1098/rspb.2003.2647.

Symonds, M.R.E., and M.A. Elgar. 2004. Species overlap, speciation and the evolution of aggregation pheromone in bark beetles. *Ecology Letters* 7:202-212.

Tanaka, A. 2003. Chemical ecology of the white pine weevil in British Columbia. Masters of Pest Management thesis, Department of Biological Sciences, Simon Fraser University, Burnaby, B. C.

Taylor, K.L. 1981. The *Sirex* woodwasp: ecology and control of an introduced forest insect. *In* The Ecology of Pests. Some Australian Case Histories. R.L. Kitching and R.E. Jones, editors. CSIRO Australia, Melbourne, Australia.

Teale, S.A., F.X. Webster, A. Zhang, and G.N. Lanier. 1991. Lanierone: a new pheromone component from *Ips pini* (Coleoptera: Scolytidae) in New York. *Journal of Chemical Ecology* 17:1159-1176.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. "The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools." Nucleic Acids Research 25: 4876-4882.

Tømmerås, B.Å., and H. Mustaparta. 1989. Single cell reponses to pheromones, host and non-host volatiles in the ambrosia beetle *Trypodendron lineatum*. *Entomologia Experimentalis et Applicata* 52:141-148.

Tumlinson, J.H., C.E. Yonce, R.E. Doolittle, R.R. Heath, C.R. Gentry, and E.R. Mitchell. 1974. Sex pheromones and reproductive isolation of the lessor peachtree borer and the peachtree borer. *Science* 185:614-616.

Upadhyay, H.P. 1981. A Monograph of *Ceratocystis* and *Ceratocystiopsis*. The University of Georgia Press, Athens, Georgia.

Uzunovic, A. 1996. Biology of blue stain in the context of modern forestry. Ph.D thesis, Department of Biology, Timber Technology, Imperial College of Science, Technology and Medicine. University of London and Imperial College, London.

Uzunovic, A., K.A. Seifert, S.H. Kim, and C. Breuil. 2000. *Ophiostoma setosum*, a common sapwood staining fungus from western North America, a new species of the *Ophiostoma piceae* complex. *Mycological Research*. 104:486-494.

Uzunovic, A., and J.F. Webber. 1998. Comparison of blue stain fungi grown *in vitro* and in freshly cut pine billets. *European Journal of Forest Pathology* 28:323-334.

Uzunovic, A., A. Yang, D.Q. Gagné, C. Breuil, L. Byrne, A. Gignac, and S.H. Kim. 1999. Fungi that cause sapstain in Canadian softwood. *Canadian Journal of Microbiology* 45:914-922.

van den Dool, H., and P.D. Kratz. 1963. A generalization of the retention index sysytem including linear temperature program. *Journal of Chromatography* 2:463-471.

Via, S., A.C. Bouck, and S. Skillman. 2000. Reproductive isolation between divergent races of pea aphids on two hosts. II. Selection against migrants and hybrids in the parental environments. *Evolution*. 54:1626-1637.

Viiri, H. 1997. Fungal associates of the spruce bark beetle *Ips typographus* L. (Col. Scolytidae) in relation to different trapping methods. Journal of Applied Entomology 121:529-533.

Vilgalys, R., and M. Hester. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* 172:4238-4236.

Vité, J.P., and A. Bakke. 1979. Synergism of chemical and physical stimuli in host colonization by an ambrosia beetle. *Naturwissenchaften* 66:528-529.

Vité, J.P., and W. Francke. 1976. The aggregation pheromones of bark beetles: Progress and problems. *Naturwissenchaften* 63:550-555.

Vité, J.P., and D.L. Williamson. 1970. *Thanasimus dubius*: Prey perception. *Journal of Insect Physiology* 16:233-239.

Webb, J.W., and R.T. Franklin. 1978. Influence of phloem moisture on brood development of southern pine beetle (Coleoptera: Scolytidae). *Environmental Entomolology* 7:405-410.

Webber, J.F., and J.N. Gibbs. 1989. Insect dissemination of fungal pathogens of trees. *In* Insect-Fungus Interactions. N.M. Wilding, N.M. Collins, P.M. Hammond, and J.F. Webber, editors. Academic Press, London.

White, T.J., T.D. Bruns, S. Lee, and J.W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* PCR Protocols: A Guide to Methods and Applications. M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, editors. Academic Press, San Diego, California.

Whitney, H.S. 1971. Associations of *Dendroctonus ponderosae* (Coleoptera: Scolytidae) with blue stain fungi and yeast during brood development in lodgepole pine. *The Canadian Entomologist* 103:1495-1503.

Whitney, H.S. 1982. Relationship between bark beetles and symbiotic organisms. *In* Bark Beetles in North American Conifers: A System of the Study of Evolutionary Biology. J.B. Mitton and K.B. Sturgeon, editors. University of Texas Press, Austin, Texas.

Wilson, E.O. 1971. The Insect Societies. Belknap, Cambridge,Massachusetts.

Wingfield, M.J., K.A. Seifert, and J.F. Webber. 1993. *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. American Phytopathogical Society Press, St. Paul, Minnesota.

Wood, D.L. 1982. The role of pheromones, kairomones, and allomones in the host selection and colonization behavior of bark beetles. *Annual Review of Entomology* 27:411-446.

Wood, D.L. and D.E. Bright. 1992. A catalog of Scolytidae and Platypodidae (Coleopera) 2. Taxonomic index. 1-1533 pp.

Wood, S.L. 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. Great Basin Naturalist Memoirs. 6: pp. 1-1359.

Yamaoka, Y., R.H. Swanson, and Y. Hiratska. 1990. Inoculation of lodgepole pine with four blue-stain fungi associated with mountain pine beetls, monitored by a heat pulse velocity(HPV) instrument. *Canadian Journal of Forest Research* 20:31-36.

Zabel, A., and J.M. Morrell. 1992. Wood Microbiology: Decay and its Prevention. Academic Press, New York.

Zar, J.H. 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, NJ.

Zhang, Q.-H., and F. Schlyter. 2004. Olfactory recognition and behavioural avoidance of angiosperm nonhost volatiles by conifer-inhabiting bark beetles. *Agricultural and Forest Entomology* 6:1-19.

Ziller, W.G. 1974. The tree rust of Western Canada, *Department of the Environment, Canadian Forest Service,* Victoria, British Columbia, *Publication No. 1329.*