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VALUE OF CUTICULAR HYDROCARBONS FOR IDENTIFYING MORPHOLOGICALLY SIMILAR SPECIES OF PINE CONE BEETLES

by

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SUMMARY

Cuticular hydrocarbons are evaluated as a suite of taxonomic characters for six species of Conophthorus: C. ponderosae, C. radiatae, C. cembroides, C. coniperda, C. resinosa, and C. banksianae. We identified a total of 69 individual and isomeric mixtures of hydrocarbons. Hydrocarbon components consist of n-alkanes, alkenes, alkadienes, 2- or 4-methylalkanes, 3-methylalkanes, single component and isomeric mixtures of internally-branched monomethylalkanes, dimethylalkanes and trimethylalkanes. Hydrocarbon mixtures in three geographically-separated populations of C. ponderosae from three different pine species are qualitatively the same with the exception of a homologous series of 3,7-dimethylalkanes from C. ponderosae collected from Pinus lambertiana. This implies that sibling species may exist in the C. ponderosae complex. Hydrocarbon patterns of C. resinosae and C. banksianae appear to be qualitatively identical; no unique hydrocarbon components were found. This supports the concern that C. banksianae is not a valid species. The results of this preliminary study indicate that cuticular hydrocarbons could be used to separate species of Conophthorus. To be useful as chemotaxonomic characters, hydrocarbons should be abundant, not minor components. They should also be unique or present in only a few of the species, or conversely, they should be common in most of the species, yet completely absent, rare, or of insignificant quantities in one or a few. Furthermore, they should have a unique elution time so that they do not coelute with another hydrocarbon in the same species nor should they elute at a time similar to a different hydrocarbon in a different species. Hydrocarbons which meet these criteria were found in five of the six Conophthorus species.

KEY WORDS--Cuticular lipids, chemotaxonomy, methyl-branched hydrocarbons, mass spectra, Conophthorus species, Pinus species, Scolytidae, insect integument.

INTRODUCTION

Conophthorus Hopkins (family Scolytidae) is a taxonomically refractory genus that is destined to increase in importance as an economic pest as production forestry in northern and western North America becomes more reliant on pine seed orchards. Future pest management strategies for these beetles will rely on control strategies or monitoring techniques based on species-specific behavior of adults. Without proper identification of species or a clear understanding of the interaction between hosts and Conophthorus species, development of a viable pest management program will not be possible.

Adult Conophthorus characteristically attack and kill female cones of Pinus. In severe infestations nearly all of the cones may be destroyed (Furniss and Carolin 1977). Such sizable reductions in seed crops can adversely affect reforestation, whether the stands are naturally regenerated or planted with seedlings. With few exceptions female beetles bore into second-year cones from May to July. One species, C. banksianae McPherson, exclusively infests twig terminals. Other species survive in twigs when cones are not available. Female beetles initiate attack through the cone stalk or through the side of the cone near its base. They rapidly girdle the axis of the cone, thereby severing the conductive tissue and killing the cone. Infested cones remain on the tree or drop to the ground. Larvae mine the cone, complete their development, and overwinter in the same cone. In some species, these brood adults may remain in diapause for an additional year (Williamson et al. 1966; Jenkins 1982).

The genus Conophthorus is restricted to North America. Keys to the species utilize morphological features of the elytra and pronotum (Wood 1982). Wood (1982) notes that "character diversity in this genus is limited. Characters are poorly developed and variable, making the separation of some species extremely difficult." Host species can be used, and in fact are used almost exclusively, to identify beetle species (Hedlin et al. 1980). Most species of Conophthorus are apparently monophagous. C. ponderosae Hopkins is a notable exception to this generalization. This species apparently breeds in 13 species of Pinus and ranges from northern British Columbia to southern Mexico. In a recent revision of the genus, Wood (1982) synonymized C. ponderosae with five other species of Conophthorus: C. scopulorum Hopkins, C. contortae Hopkins, C. monticolae Hopkins, C. flexilis Hopkins, and C. lambertianae Hopkins. He based his revision on the holotypes of all the above species and 784 other specimens. He concluded "...the variability in this material is difficult to interpret. It is entirely possible that two or more sibling species are represented."

During speciation species normally also develop morphological differences which we, as biologists, use to identify them. Some species fail to acquire conspicuous morphological differences during the process of speciation and are called sibling species (Mayr 1969). Sibling species are obviously inconvenient to museum taxonomists, however, they also pose significant problems for forest biologists, including pest management specialists. Correct taxonomic classification is fundamental to understanding all biological processes.

Since the different species of Conophthorus are difficult to separate morphologically, we decided to evaluate cuticular hydrocarbons as another suite of taxonomic characters for species determination. It seems incongruous that only one species of Conophthorus would be polyphagous. It is possible that C. ponderosae is in the process of speciation or that there are true biological (albeit sibling) species awaiting discovery. Our overall goal is to examine the taxonomic status of all species of Conophthorus by evaluating a set of characters other than morphological features. We have chosen to examine the cuticular hydrocarbons as a possible additional set of taxonomic characters.

Insect Hydrocarbons

The surface of all terrestrial insects is covered with a thin layer of wax that increases their fitness (Hadley 1985). This wax plays a key role in survival of the insect by providing protection from dessication (Hadley 1982), as well as serving as a barrier to abrasion, microorganisms, and chemicals (Blomquist and Dillwith 1985; Jackson and Blomquist 1976). Some wax components have been shown to serve a major function in chemical communication as pheromones and kairomones (Howard and Blomquist 1982). Hydrocarbons are the predominant lipids in insect waxes.

Alkanes occur in all the insect surface lipids so far investigated. The n-alkanes generally range from 21 to 36 carbons atoms with alkanes having an odd number of carbons predominating. 2-Methylalkanes and/or 3-methylalkanes are also prevalent in insect surface lipids. Like the n-alkanes, the terminally-branched monomethylalkanes range from simple compositions where only one compound is present to complex mixtures of 2-methylalkanes, 3-methylalkanes or mixtures of 2- and 3-methylalkanes. The 2-methylalkanes are somewhat unique in that components with both odd- and even-numbered carbon chains are present in substantial amounts, which reflects their biosynthetic origin from the carbon skeleton of valine and leucine (Blomquist and Dillwith 1985).

The majority of the internally-branched monomethylalkanes have the methyl branch located on an odd-numbered carbon atom between carbons 5 and 17. Monomethylalkanes with the methyl branch on even-numbered carbon atoms are usually minor components. Many of the long-chain, internally-branched dimethylalkanes have an isoprenoid-type spacing, although they are not derived from isoprenoid units. Common isomers are 9,13-; 11,15-; 13,17-; 15,19- and 17,21-. Methyl branches on many other positions have been reported. As careful analyses of dimethylalkanes are made on more species, it appears that the methyl groups can be positioned almost anywhere on the chain, although usually on odd-numbered carbons. n-Alkenes, with one, two, or three double bonds, have been characterized from about one-half of the insect species examined to date. The chain length of cuticular n-alkenes usually ranges from 20 to 37 carbon atoms, with odd-numbered chain lengths predominating. The position of double bonds can be almost anywhere in the chain but is common at the 9 carbon (Blomquist and Dillwith 1985).

Hydrocarbons as Taxonomic Characters

Insects synthesize most if not all of their compliment of cuticular hydrocarbons de novo (Jackson and Blomquist 1976; Blomquist and Dillwith 1985). We can assume this synthesis is genetically controlled and affected only slightly by environmental parameters. In certain groups of insects which include termites, bees, mosquitoes, cockroaches and beetles, cuticular hydrocarbon profiles appear to have promise as taxonomic characters (Carlson and Bolten 1984; Carlson and Service 1980; Gastner and Nation 1986; Howard and Blomquist 1982; Vander Meer 1986). Howard et al. (1978) and Blomquist et al. (1979) were among the first to recognize the value of hydrocarbons as taxonomic characters. They found that the termites Reticulitermes flavipes (Kollar) (Rhinotermitidae) and Zooptermopsis angusticollis (Hagen) (Termopsidae) possess drastically different hydrocarbon profiles, and both of these differ markedly from those reported earlier for Nasuititermes exitiosus (Hill) (Nasuititermitidae) (Moore 1969). In addition, Howard and his colleagues characterized the cuticular hydrocarbons of R. virginicus (Banks) and partially characterized the hydrocarbons of five other species of Reticulitermes (Clément et al. 1985; Howard et al. 1982). In every case, hydrocarbon composition was unique.

The most thorough examination of cuticular hydrocarbons as species characters in the Coleoptera has been made by Lockey (1978, 1979a,b, 1980, 1981, 1982a,b,c). He compared the cuticular hydrocarbons of nine tenebrionid species. He found that each species has distinctive hydrocarbon compositions and congeneric species have hydrocarbon mixtures which are more similiar than those of more distantly-related species. He was able to discern a pattern in hydrocarbon mixtures of selected tenebrionid beetles which paralleled their grouping into the tribes Tenebrionini and Diaperini.

We have expanded on the early work of Blomquist et al. (1979) and characterized the cuticular hydrocarbons of the entire genus of dampwood termites, Zootermopsis (Haverty et al. 1988). We were able to correlate hydrocarbon "signatures" with a heretofore undescribed mandibular character, shape and size of the subsidiary tooth, to identify the three presently recognized species of Zootermopsis (Thorne and Haverty, In press). Furthermore, these hydrocarbon studies allowed us to recognize a new subspecies (or possibly species) of Zootermopsis. The validity of this taxon has also been implicated by studies of agonistic behavior (Haverty and Thorne, In press).

With our success in characterizing an entire genus of termites and the growing acceptance of hydrocarbons as taxonomic characters, we decided to evaluate cuticular hydrocarbons as another suite of characters in Conophthorus. In this preliminary report we examined the hydrocarbon mixtures of six species: C. ponderosae, C. radiatae Hopkins, C. cembroides Wood, C. coniperda (Schwarz), C. resinosae Hopkins, and C. banksianae. This is the beginning of an extensive investigation to determine the degree of similarity and/or diversity among species in the genus Conophthorus in North America based on cuticular hydrocarbons.

METHODS AND MATERIALS

Adult cone beetles were collected as overwintering adults from infested cones or twig terminals. Only C. ponderosae was collected from three different species of pine (Pinus ponderosa, P. lambertiana, and P. monticolae) from three different locations. Beetles were allowed to emerge from the cones or twigs. One to three days after emergence they were frozen and held at -20°C until hydrocarbons were extracted. Beetles were removed from the freezer and allowed to warm to ambient temperature. Beetles shipped from the eastern half of North America were air-dried. Cuticular lipids were extracted by immersing 15 to 50 beetles, as a group, in 10 ml of hexane for 10 min. After extraction, hydrocarbons were separated from other components by pipetting the 10-ml extract and an additional 8 ml of hexane through 3 cm of activated BioSil-A in Pastuer pipette mini-columns (Blailock et al., 1976; Haverty et al., 1988). All hydrocarbon extracts were evaporated to dryness under a stream of nitrogen and redissolved in 30 ul of hexane for GC analyses.

Gas chromatography-mass spectrometry (GC-MS) analyses were performed on a Hewlett Packard 5890 gas chromatograph equipped with a Hewlett Packard 5970B Mass Selective Detector interfaced with Hewlett Packard Chemstation computer. The GC-MS was equipped with a fused silica capillary column (30 m x 0.2 mm ID, HP-1) and operated in split mode (with a split ratio of 20:1). Each mixture was analyzed by a temperature program from 200°C to 320° at 3°C/min with a final hold of 20 minutes. Electron impact (EI) mass spectra were obtained at 70 eV. n-Alkanes were identified by comparing their retention times and mass spectra with external standards. Alkenes and methyl-branched alkanes were tentatively identified by calculating their equivalent chain lengths (Nelson and Sukkestad 1970; Jackson and Blomquist 1976). Mass spectra of methlyalkanes were interpreted as described by Nelson et al. (1972), Nelson (1978), Ponomis et al. (1978), and Blomquist et al. (1987).

RESULTS

We identified a total of 69 major individual and isomeric mixtures of hydrocarbons (mean percent $\geq$ 0.5% of the total hydrocarbon mixture) in the cuticular wax of six species of Conophthorus. The hydrocarbon components consist of n-alkanes, alkenes, alkadienes, 2- or 4-methylalkanes, 3-methylalkanes, single component and isomeric mixtures of internally-branched monomethylalkanes, dimethylalkanes and trimethylalkanes. Unique and abundant, species-specific hydrocarbon components were identified.

The normal alkane composition in all six species is a continuous series from n-heneicosane to n-tritriacontane. n-Tricosane, n-pentacosane and n-heptacosane predominate. All six species have measurable quantities of normal alkanes of even-numbered chain length from n-docosane to n-octocosane. n-Tetracosane and n-hexacosane are the most abundant even-numbered normal alkanes. With the exception of n-hentriacontane, these species contain all of the same n-alkanes components from n-heneicosane to

n-tritriacontane. C. resinosae and C. banksianae, however, contain significant amounts (≤ 1.0%) of n-hentriacontane. Usually n-alkanes have little taxonomic value because nearly all species have these components in their hydrocarbon mixtures. However, we feel n-hentriacontane could be used for species discrimination in Conophthorus.

The Conophthorus species examined in this report all have the unsaturated hydrocarbons, pentacosene and heptacosene. Other alkenes are present in very small, or trace, amounts and occur only as a shoulder of the peak of a terminally-branched monomethyl alkane. C. resinosae and C. banksianae both contain significant amounts of pentatriacontene and heptatriacontene. These hydrocarbons elute at unique times (ECL = 34.7 and 36.7, respectively) and may prove to be diagnostic for these species since they are not found in any of the other four species. C. ponderosae and C. cembroides do not contain significant quantities of alkadienes. Pentacosadiene in C. ponderosae and C. radiatae and heptacosadiene in C. coniperda coelute with a methyalkane; this makes them difficult to use for diagnostic purposes.

Terminally-branched alkanes are relatively rare in these six species of Conophthorus. Virtually none were found in C. cembroides. Significant quantities of internally-branched monomethylalkanes occur in these Conophthorus species as complex isomeric mixtures with methyl branches on odd-numbered carbons 9, 11, 13 and 15. Dimethylalkanes in insects generally have methyl branches on odd-numbered carbons separated by an odd number of carbons (Blomquist et al. 1987). All of the dimethylalkanes produced by these species of Conophthorus fit this pattern. The majority of the methyl branches are separated by three carbons and include the 3,7-dimethyalkanes (C. ponderosae from sugar pine), the 11,15- and 13,17-dimethylalkanes (C. ponderosae, C. cembroides) and 15,19-dimethypentatriacontane (C. ponderosae, C. radiatae). C. coniperda, C. resinosae and C. banksianae contain only small quantities of dimethylalkanes in their hydrocarbon mixtures, whereas C. cembroides, C. ponderosae and C. radiatae all contain significant quantities of dimethylalkanes. The hydrocarbon mixtures of C. cembroides contain significant amounts of 9,15- and 11,15-dimethylheptacosane and 11,15-; 13,17-dimethylnonacosane. These unique, species-specific dimethyalkanes with unique ECLs are not found in the other five species. We identified a homologous series of 13,17,23-trimethlyalkanes starting at the C_{36} carbon (ECL = 33.9) in C. ponderosae, C. radiatae, and possibly C. resinosae and C. banksianae. C. coniperda and C. cembroides clearly contain fewer trimethylalkanes than the other four species of Conophthorus with trimethylalkanes reported in this paper.

DISCUSSION

The cuticular hydrocarbons of the six species of Conophthorus reported in this paper, as is the case for many insect species (Blomquist et al. 1987), consist of complex mixture of straight-chained and methyl-branched, saturated components and one or more unsaturated components. The relatively large number of hydrocarbon components often found on the cuticle of insects, ease of chemical analysis and identification, and species-specific

compositions for many insects make them attractive characters for use in chemotaxonomy (Carlson and Bolten 1984; Gastner and Nation 1986; Haverty et al. 1988; Vander Meer 1986).

Cuticular hydrocarbons, particularly the unsaturated and methyl-branched components, are biosynthesized by insects (Blomquist et al. 1987). A small percentage of the n-alkanes in several species (Blomquist and Jackson 1979; Nelson et al. 1971) may arise from the diet, but unsaturated and methy-branched hydrocarbons are rare in plants. Thus, the prevalence of unsaturated and methyl-branched components in Conophthorus is fortuitous. Although it has been clearly shown that the methyl branch groups of the 3-methyl and all internally branched alkanes arise form the insertion of methylmalonyl-CoA in place of malonyl-CoA during chain elongation, it is not known how the positions and number of methyl branches are regulated to produce, what appears to be in many cases, species-specific cuticular hydrocarbon profiles. In some insects, the amino acid valine (and presumably methionine and isoluecine) serves as the precursor to the carbon skeleton of methylmalonyl-CoA (Dillwith et al. 1982; Halarnkar et al. 1985), whereas in other species succinate is converted to methymalonyl-CoA (Blomquist et al. 1980; Chu and Blomquist 1980). Regardless of the precursors and regulation, the end products appear sufficiently diverse to serve as chemotaxonomic characters in many hard-to-distinquish species.

Conophthorus is a refractory genus. Many of the species are difficult to separate on the basis of morphological characters alone. We hope to develop a new suite of characters for the entire genus, similar to our study with the dampwood termites, Zootermopsis (Haverty et al. 1988). Thus far the results are encouraging. The cuticular hydrocarbon mixtures contain diagnostic n-alkanes, alkenes, alkadienes and monomethyl-, dimethyl- and trimethylalkanes, which are useful for separation of these species.

The composition of the major cuticular wax components in three populations of Conophthorus ponderosae is complex. With one possible exception, the hydrocarbon mixtures are qualitatively the same in all three of the populations we examined: 3,7-dimethylalkanes in C. ponderosae from sugar pine. It may be possible the C. ponderosae populations we collected from ponderosa pine and western white pine produce these dimethylalkanes in insignificant quantities. Future analyses with more adults from additional populations may resolve the presence or absence of these 3,7-dimethylalkanes in C. ponderosae. If one agrees that hydrocarbon profiles are species-specific (Howard et al. 1982; Haverty et. al. 1988; Vander Meer 1986), we would infer that two populations of C. ponderosae evaluated here are the same species, or at least very closely related. C. ponderosae from these three pine species may be sibling species, subspecies or distinct populations of the same species whose cuticular hydrocarbon phenotypes have drifted apart in response to evolutionary pressures such as host, climate or geographical location.

The hydrocarbon patterns of C. resinosae and C. banksianae are qualitatively identical; no unique hydrocarbon components were found between these two species. They do possess several components which easily separate them from the other four species discussed in this paper. This evidence supports the concern that C. banksianae is not a valid species (Stephen

Wood, personal communication). Further elucidation with biological and genetic studies will be necessary to resolve this problem.

The results reported in this paper are preliminary in that we have not characterized the cuticular hydrocarbons of all of the extant species of Conophthorus nor have we examined numerous populations of each species. We could, with the data available, develop a dichotomous key to these six species. We feel it would be premature at this point in our research. We can illustrate the potential or value of cuticular hydrocarbons for developing such keys by selecting the hydrocarbons we might use (Table 1). Useful hydrocarbons should be abundant, not minor components (at least 1%, but preferably > 5% of the total hydrocarbon mixture). They should also be unique or present in only a few of the species, or conversely, they should be common in most of the species yet completely absent, rare or of insignificant quantities in one or a few. Furthermore, they should have a unique elution time so that they do not coelute with another hydrocarbon in the same species nor should they elulte at a time similar to a different hydrocarbon in a different species. From a collection of these hydrocarbons, many different dichotomous keys are possible.

Future studies will involve further characterization of the cuticular hydrocarbons of additional, morphologically-distinct species of Conophthorus, and quantitative evaluation of intra- and interspecific variation. Hopefully, these studies will help clarify the taxonomy of this genus, especially the polyphagous species, C. ponderosae, and the potentially invalid species, C. banksianae and C. cembroides.

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Table 1. Diagnostic hydrocarbons identified from six species of Conophthorus.[a]

Hydrocarbon	ECL	Conophthorus species[b]					
		Cpon	Ccem	Ccon	Crad	Cres	Cban
Pentacosadiene	24.69	tr	o	o	++	++	++
Heptacosene	26.70	+	+	+++	tr	++	++
9-; 11-; 13-Methylheptacosane	27.32	+	+	+++	tr	+	+
11,15-Dimethylheptacosane +	27.63	o	+++	o	o	o	o
9,15-Dimethylheptacosane		o	++	o	o	o	o
15-Methyloctacosane	28.32	o	o	++	o	o	o
9-; 11-; 13-; 15-Methylnonacosane	29.32	tr	o	++	o	o	o
11,15-; 13,17-Dimethylnonacosane	29.61	o	+++	o	o	o	o
n-Hentriacontane	31.00	+	++	o	o	++	++
11-; 15-Methyltritriacontane	33.30	++	++	x	tr	o	o
Pentatriacontene	34.70	tr	o	o	o	++	++
11-; 13-Methylpentatriacontane	35.31	++	++	o	++	+	+
Heptatriacontene	36.70	o	o	o	o	+++	+++
13,17,21-Trimethylheptatriacontane	37.90	++	o	x	+	++	++
11-; 13-Methylnonatriacontane	39.30	++	++	o	++	+	+
13,17,23-, 13,17,25-Trimethyl	39.90	++	o	o	++	x	x
nonatriacontane							

[a] A triple + indicates $\geq$ 5.0% , ++ from 1.0 to 5.0% and + from 0.5 to 1.0% of the total hydrocarbon component. Some trace (tr.) components appear infrequently or consistently in very small quantities (< 0.5% of the total). A zero indicates the hydrocarbon was never identified for the species. Compounds with an x were not identified for the species; mass spectra were insufficient to make a positive identification of the hydrocarbon.

[b] Cpon = Conophthorus ponderosae, Ccem = C. cembroides, Ccon = C. coniperda, Crad = C. radiatae, Cres = C. resinosae, and Cban = C. banksianae.

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