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Cuticular Hydrocarbon Profiling of Australian Gonipterini Weevils

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Abstract: Cuticular hydrocarbon (CHC) profiling shows promise as a chemotaxonomic tool for identifying and discriminating between closely related insect species. However, there have been limited studies using CHC profiling to differentiate between weevil species (Coleoptera: Curculionidae). This proof-of-concept study investigated the use of CHC and volatile profiling to discriminate between five weevil species from three genera in the Gonipterini tribe. A total of 56 CHCs and 41 other volatile compounds were found across the five species, with 83 of the compounds being identified through their mass fragmentation patterns. The number of CHCs from each species ranged from 20 to 43, while the proportion of CHCs unique to each species varied between 0% and 19%. The most abundant CHCs were nonacosane, 7-methylheptacosane, heptacosane, and hexacosane. Principal component analysis of the centred log-ratio transformed data revealed broad differences in CHC profiles between the two *Oxyops* species, with *Bryachus squamicollis* demonstrating the greatest divergence from the other Gonipterini species. The results suggest that CHC analysis could be used to support established taxonomic methods, including morphological features and genetic sequencing results.

Keywords: *Gonipterus*; *Oxyops*; *Eucalyptus*; chemotaxonomy

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1. Introduction

Traditionally, insect taxonomy has been based on morphological features [1,2]. In the last few decades, genetic techniques such as DNA sequencing and barcoding have emerged as significant taxonomic tools [3,4]. Another complementary taxonomic technique is chemotaxonomy—the use of differences in biochemical composition between species to classify and/or identify them [5,6]. Originally used for the classification of plant species [7], the technique was subsequently extended to other organisms, such as insects. The major focus has been on cuticular hydrocarbons (CHCs) [8], which are found on the cuticles of virtually all insects, act to prevent desiccation, and serve as signalling molecules for communicating with other insects. CHCs are synthesised by the insect through a number of inter-linked anabolic pathways; hence, they are reflective of the genetic diversity and metabolic pathways of the species [9,10]. For several decades, CHC profiling has been used to classify various insect species [11,12]; however, it should be noted that this method is not without its drawbacks. These include high levels of intra-specific variation in some cases, environmental variation, and the challenges of defining CHC boundaries between species [11].

There have been a limited number of studies performed on the cuticular hydrocarbon profiles of weevils (Coleoptera: Curculionidae), despite the extensive diversity and ecological significance of this family. One early study by Baker and Nelson [13] investigated the cowpea weevil (*Callosobruchus maculatus*), finding that mono- and dimethyl branched-chain alkanes comprised the majority of CHCs in this species, with no difference in CHC profiles between sexes. Similarly, Lapointe et al. [14] investigated the Diaprepes root weevil (*Diaprepes abbreviatus*) and found no significant differences by sex or maturity stage. However, observations by Martins et al. [15] suggested that males of the rice water weevil (*Oryzophagus oryzae*) recognise females from their CHC profiles, indicating that some differentiation must be possible.

Finally, Souza et al. [16] recently demonstrated that the cuticular hydrocarbon profiles of several species of *Gonipterus* weevil agreed well with molecular sequencing data, suggesting that CHC profiling could be used for the accurate classification of species from this genus. These species are from the Gonipterini tribe, which encompasses the genera *Bryachus* (Pascoe 1870), *Gonipterus* (Schoenherr 1833), *Iptergonus* (Lea 1908), *Oxyops* (Schoenherr 1826), *Pantoreites* (Pascoe 1870), *Prophaesia* (Pascoe 1870), and *Syarbis* (Pascoe 1865). This tribe is native to the Australo-Pacific region, although some species (particularly *Gonipterus* spp.) have been accidentally translocated to various locations worldwide [17]. Both adults and larvae feed on *Eucalyptus* leaves. Outside of their native range, several species of *Gonipterus* have become significantly destructive pests of commercial *Eucalyptus* plantations [18] due to the absence of its natural parasitoids—principally, *Anaphes nitens* [19]. The *Gonipterus* genus in particular contains a number of cryptic species [17], which has posed significant barriers to the success of biocontrol programs [18]. Identification of such species typically requires molecular analysis and dissection of male genitalia [17].

If successful, rapid CHC profiling could provide an alternative to costly and time-consuming molecular sequencing and/or dissection techniques for the identification of morphologically cryptic species from this economically important tribe. Hence, this proof-of-concept study aimed to extend the CHC profiling method of Souza et al. [16] to discriminate between different Gonipterini genera, as well as between different species in specific genera (*Gonipterus* and *Oxyops*).

2. Materials and Methods

2.1. Specimen Collection

Fifteen weevils were hand collected from *Eucalyptus populnea* Muell. saplings in Central Queensland (23°46′ S, 150°21′ E) on 14 February 2021. They comprised five specimens of *Oxyops fasciculatus* Redtenbacher, three of an undescribed *Oxyops* sp. only known from this location (designated throughout this manuscript as *Oxyops* sp. 1), one specimen tentatively identified as *Gonipterus* sp. n. 2, three of *Gonipterus cinnamomeus* Pascoe, and three of *Bryachus squamicollis* Pascoe. Due to the limited number of Gonipterini weevils found during the fieldwork, a larger sample size was not possible for some species. Species delineations were confirmed by Dr Rolf Oberprieler (CSIRO, Canberra, Australia).

2.2. Extraction of CHCs

The CHC extraction methods followed those of Souza et al. [16]. After being killed in a freezer (−20 °C), each weevil was placed in a 2.0 mL GC-MS vial along with 300 μL of hexane. After 4 min, they were agitated for one minute by using a vortex mixer, and the hexane extract was collected. The weevil specimens were subsequently preserved in 100% ethanol.

2.3. Analysis of CHCs

The hexane extracts were analysed via gas chromatography-mass spectrometry (GC-MS) while following the methods of Souza et al. [16]. Analysis was performed on a single-quadrupole Shimadzu QP2010 Plus system (Shimadzu, Kyoto, Japan) fitted with an autoinjector/autosampler (AOC-20i/s) and Shimadzu SH-Rxi-5Sil MS column (29 m × 0.25 mm i.d. × 0.25 μm thickness). The following conditions were used: carrier gas—helium at 1.93 mL min^{-1}, injection temperature—250 °C, injection volume—1 μL, split ratio 5:1, ion source temperature—230 °C, interface temperature—230 °C, MS mass range—35–600 *m*/*z*, and scan rate—3.3 scans/sec. During the run, the column temperature was initially held at 40 °C for 2 min before ramping linearly at 10 °C/min to reach 260 °C, where it was held for a further 6 min. The total run time was 30 min. Higher temperatures were not used in this study, as the main focus was on low- to moderate-weight CHCs, as studied by Souza et al. [16].

Chromatogram peaks were integrated if they had a peak area of >10,000 units and slope of >1000 units/min. Linear retention indices (LRIs) were calculated from the retention times of alkane standards (C_8–C_{40}) run under the same conditions [20]. Compound identities were established through the comparison of their mass spectra and LRIs with the NIST14 and NIST14s libraries and the relevant literature [14,21–23].

2.4. Chemometric Analysis

Data analysis of the volatile compound abundance was conducted in R Studio running R 4.0.5. [24]. Where applicable, the results are presented as the mean ± 1 standard deviation. The CHC dataset was transformed by using the centred log-ratio (clr) method prior to principal component analysis (PCA).

3. Results

3.1. Cuticular Hydrocarbon Profiles

Typical GC-MS chromatograms obtained for each species are provided in Figure 1, while Table 1 shows the compounds identified across all Gonipterini species. A total of 97 peaks were found across all species, with 59 compounds being able to be positively identified from their mass fragmentation patterns and LRIs. A further 24 compounds were tentatively identified, with 14 compounds (10 alkanes, 3 ketones, and 1 aldehyde) being unable to be precisely identified. Some of the compounds identified (e.g., eucalyptol and globulol) appeared to be derived from the host plants (*E. populnea*), rather than being synthesised by the weevils. However, the majority of the compounds (56) could be classified as CHCs (Table 1). While all compounds are discussed in this section, only the CHC data were used in the subsequent chemometric analysis.

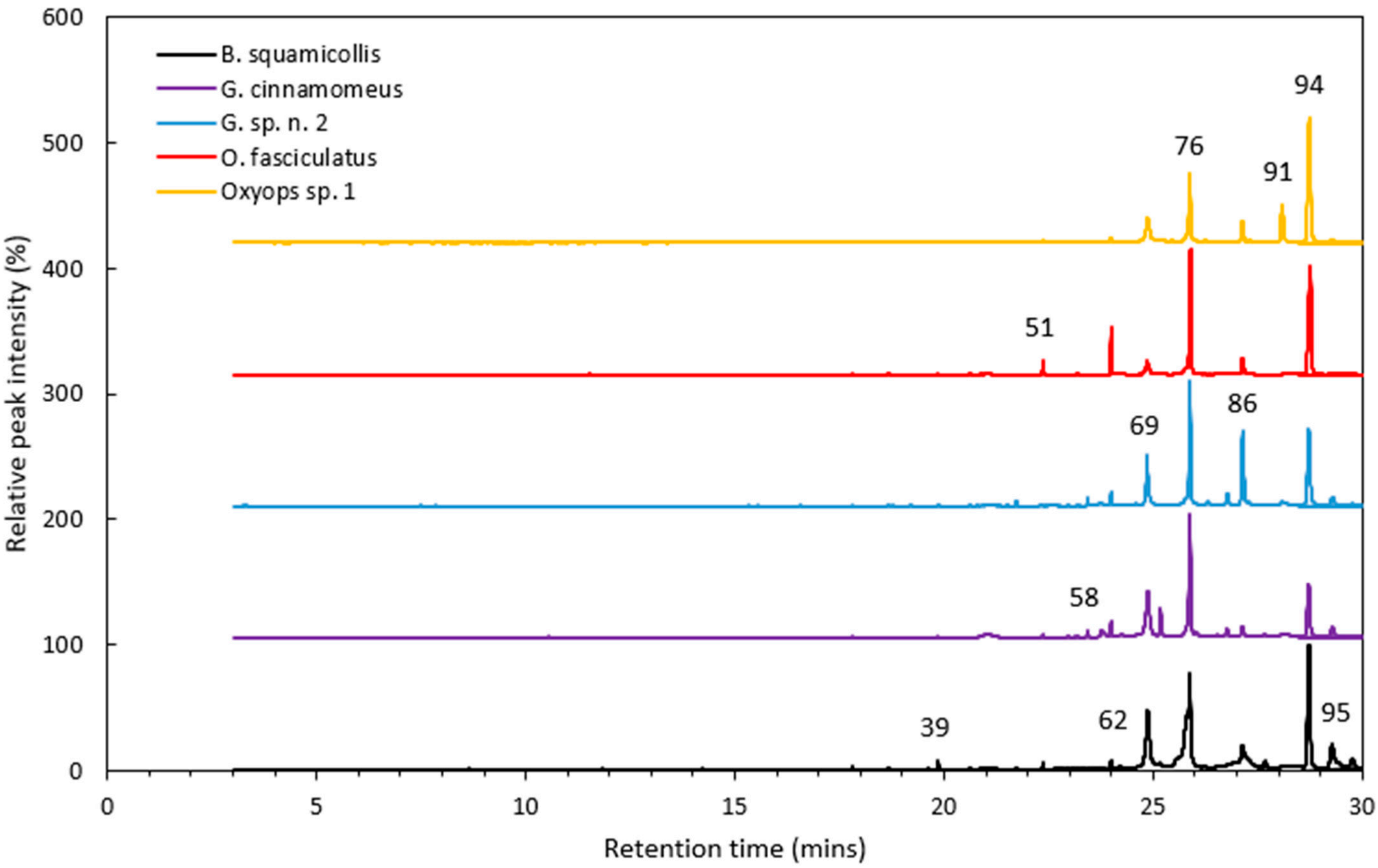

Figure 1. GG-MS chromatogram of the hexane extracts from each Gonipterini species. The major peaks are marked; the compound numbers correspond to those provided in Tables 1 and 2.

Table 1. Identification details for the compounds found in the Gonipterini hexane extracts. Compounds classified as CHCs are highlighted in bold.

No.	Compound	Class	LRI Rxi-5Sil	Lit. LRI	M+ (*m*/*z*)	Other Confirmatory MS Peaks (*m*/*z*)	Ident. ^	Roles # (Coleoptera)	Roles # (Other Insects)
1	3-hexanone	Ketone	787	789	100	43, 57, 71	MS, LRI	-	-
2	2-hexanone	Ketone	791	793	100	43, 58, 85, 71	MS, LRI	-	-
3	**2,4-dimethylheptane**	**Dimethyl alkane**	**820**	**822**	**128**	**43, 85, 57, 71**	**MS, LRI**	**-**	**-**
4	Heptanal	Aldehyde	902	902	114	70, 44, 55, 57, 81, 86, 96	MS, LRI	A, Al	A, Al, K, P
5	Octanal	Aldehyde	1002	1001	128	57, 56, 84, 69, 95, 100, 110	MS, LRI	A, Al	A, Al, K, P
6	Eucalyptol	Monoterpenoid	1033	1033	154	81, 108, 139, 93	MS, LRI	A, Al, P	A, K, P
7	**3,6-dimethyldecane**	**Dimethyl alkane**	**1055**	**1086**	**170**	**57, 71, 85, 113, 127**	**MS, LRI**	**-**	**-**
8	**2,6,8-trimethyldecane**	**Trimethyl alkane**	**1099**	**1104**	**184**	**85, 99, 127, 113, 155**	**MS, LRI**	**-**	**-**
9	Nonanal	Aldehyde	1104	1108	142	57, 70, 82, 98, 95, 96, 114	MS, LRI	A, Al, P	A, Al, K, P
10	Decanal	Aldehyde	1205	1204	156	57, 70, 82, 95, 112, 128	MS, LRI	A, Al, K, P	A, K, P
11	Exo-2-hydroxycineole		1228	1228	170	108, 126, 93	MS, LRI	-	P
12	**2,6,10-trimethylundecane †**	**Trimethyl alkane**	**1275**	**1275**	**198**	**57, 71, 85, 99, 127, 113, 155**	**MS, LRI**	**-**	**-**
13	10-undecenal	Alkene aldehyde	1282	1277	168	55, 67, 81, 97, 111, 135	MS, LRI	-	-
14	Carvacrol †	Monoterpenoid	1297	1298	150	81, 93, 135, 121	MS, LRI	-	P
15	Isoascaridole †		1312	1303	168	95, 110, 81, 139	MS, LRI	-	-
16	4a-methyldecahydro-1-naphthalenol †		1319	1363	168	95, 67, 97, 135, 121	MS, LRI	-	-
17	**4,6-dimethyldodecane**	**Dimethyl alkane**	**1321**	**1325**	**198**	**85, 99, 113, 127, 155**	**MS, LRI**	**-**	**-**
18	*cis*-*p*-menth-1-en-3,8-diol †		1358	1362	170	84, 71, 109, 138	MS, LRI	-	-
19	(+)-*cis*,*trans*-nepetalactone	Iridoid	1364	1365	166	81, 95, 123, 109, 138	MS, LRI	Al	A, Al
20	Dodecanal	Aldehyde	1408	1407	184	57, 82, 96, 110, 140, 123	MS, LRI	Al	K, P
21	Aromadendrene	Sesquiterpenoid	1444	1440	204	161, 105, 133, 189	MS, LRI	-	A
22	**Unidentified hydrocarbon 1**	**-**	**1488**	**-**	**-**	**57, 71, 85, 99, 113, 127, 141, 155, 169**	**MS**	**-**	**-**
23	Bicyclogermacrene	Sesquiterpenoid	1501	1494	204	121, 161, 136, 189	MS, LRI	-	A, P
24	**2,6,10-trimethyltridecane †**	**Trimethyl alkane**	**1534**	**1540**	**226**	**99, 113, 127, 155, 141, 169**	**MS, LRI**	**-**	**-**
25	Globulol	Sesquiterpenoid	1592	1604	222	107, 109, 161, 189, 204	MS, LRI	-	A
26	Tetradecanal	Aldehyde	1612	1611	212	57, 82, 96, 124, 168	MS, LRI	Al	A, Al, P
27	**Heptadecane**	***n*-alkane**	**1699**	**1700**	**240**	**169, 183, 197**	**MS, LRI**	**A, Al, P**	**A, Al, P**
28	**Phytane**	**Branched alkane**	**1743**	**1753**	**282**	**127, 155, 169, 197, 211**	**MS, LRI**	**-**	**-**
29	*cis*-9-hexadecenal	Alkene aldehyde	1795	1800	238	55, 69, 81, 93, 111, 121, 135, 149	MS, LRI	-	A, P
30	Hexadecanal	Aldehyde	1816	1819	240	57, 82, 96, 110, 124, 138, 165, 194, 222	MS, LRI	Al, P	A, Al, P
31	6,10,14-trimethyl-2-pentadecanone	Branched ketone	1840	1842	268	58, 71, 85, 95, 109, 124, 137, 165, 250	MS, LRI	-	-
32	2-heptadecanone	Ketone	1899	1886	254	58, 71, 96, 127, 166	MS, LRI	-	-

Table 1. *Cont.*

No.	Compound	Class	LRI Rxi-5Sil	Lit. LRI	M+ (*m*/*z*)	Other Confirmatory MS Peaks (*m*/*z*)	Ident. ^	Roles # (Coleoptera)	Roles # (Other Insects)
33	**2,2-dimethyloctadecane †**	**Dimethyl alkane**	**1910**	**1917**	**282**	**127, 155, 141, 169, 183, 197, 211, 239**	**MS, LRI**	**-**	**-**
34	Heptadecanal	Aldehyde	1918	1920	254	138, 152, 166, 180, 194, 210, 226, 236	MS, LRI	-	Al, P
35	**3-ethyl-3-methylheptadecane †**	**Branched alkane**	**1953**	**1956**	**282**	**127, 141, 155, 169, 183, 197, 223**	**MS, LRI**	**-**	**-**
36	9-octadecanone	Ketone	1990	1980	268	71, 95, 141, 156, 169, 211, 254	MS, LRI	-	-
37	*cis*-13-octadecenal	Alkene aldehyde	1995	1985	266	69, 81, 83, 95, 98, 111, 121, 135, 166, 248	MS, LRI	-	A, P
38	*cis*-9-octadecenal †	Alkene aldehyde	2014	2007	266	55, 69, 96, 121	MS, LRI	P	P
39	Octadecanal	Aldehyde	2019	2021	268	124, 138, 152, 166, 180, 194, 222, 250	MS, LRI	Al, P	P
40	*cis*-2-octadecen-1-ol acetate	Ester	2074	2086	310	55, 69, 81, 97, 136	MS, LRI	-	-
41	2-nonadecanone	Ketone	2098	2101	282	58, 71, 85, 96, 100, 127, 138, 152, 267, 282	MS, LRI	-	-
42	Nonadecanal	Aldehyde	2117	2105	282	82, 96, 109, 124, 138, 152, 166, 180	MS, LRI	-	P
43	**Unidentified hydrocarbon 2**	**-**	**2128**	**-**	**-**	**211, 225, 239, 253, 267, 281, 295**	**MS**	**-**	**-**
44	**Unidentified hydrocarbon 3**	**-**	**2139**	**-**	**-**	**127, 155, 183, 211, 239, 267**	**MS**	**-**	**-**
45	**Unidentified hydrocarbon 4**	**-**	**2148**	**-**	**-**	**225, 238, 252, 267, 295**	**MS**	**-**	**-**
46	**Unidentified hydrocarbon 5**	**-**	**2160**	**-**	**-**	**155, 169, 183, 253, 197**	**MS**	**-**	**-**
47	**Unidentified hydrocarbon 6**	**-**	**2168**	**-**	**-**	**99, 127, 155, 183**	**MS**	**-**	**-**
48	**Docosane**	***n*-alkane**	**2197**	**2200**	**310**	**155, 169, 183, 196, 211, 239, 267**	**MS, LRI**	**P**	**A, Al, P**
49	Eicosanal	Aldehyde	2222	2224	296	278, 250, 152, 124	MS, LRI	Al	P
50	**Unidentified hydrocarbon 7 [a]**	**-**	**2260**	**-**	**-**	**127, 141, 155, 169, 183, 197, 211, 225, 239, 253, 267, 281**	**MS**	**-**	**-**
51	**Tricosane**	***n*-alkane**	**2297**	**2300**	**324**	**225, 239, 253, 267, 281, 295**	**MS, LRI**	**A, Al, P**	**A, Al, K, P**
52	Unidentified ketone 1	Ketone	2304	-	-	58, 59, 71, 85, 96, 127	MS	-	-
53	Henicosanal	Aldehyde	2326	2329	310	82, 96, 110, 124, 209	MS, LRI	-	-
54	**11-methyltricosane**	**Methyl alkane**	**2331**	**2330**	**338**	**99, 113, 127, 141, 155, 169, 196, 211, 239**	**MS, LRI**	**-**	**P**
55	**Unidentified aldehyde**	**Aldehyde**	**2367**	**-**	**-**	**82, 97, 109, 125, 139, 180**	**MS**	**-**	**-**
56	**3-methyltricosane**	**Methyl alkane**	**2374**	**2375**	**338**	**57, 71, 85, 96, 141, 183, 239**	**MS, LRI**	**P**	**P**
57	**Tetracosane**	***n*-alkane**	**2400**	**2400**	**338**	**267, 281, 295, 309**	**MS, LRI**	**P**	**A, Al, P**
58	Docosanal	Aldehyde	2430	2430	324	82, 96, 152, 166, 250, 278, 306	MS, LRI	P	P
59	**9-methyltetracosane †**	**Methyl alkane**	**2437**	**2433**	**352**	**99, 113, 127, 141, 155, 169, 183, 197**	**MS, LRI**	**P**	**P**

Table 1. *Cont.*

No.	Compound	Class	LRI Rxi-5Sil	Lit. LRI	M+ (*m*/*z*)	Other Confirmatory MS Peaks (*m*/*z*)	Ident. ^	Roles # (Coleoptera)	Roles # (Other Insects)
60	**2-methyltetracosane**	**Methyl alkane**	**2473**	**2465**	**352**	**309, 267, 281, 295, 337**	**MS, LRI**	**P**	**P**
61	x-pentacosene †	Alkene	2479	2477	350	168, 181, 197, 211, 225, 239, 253, 267	MS, LRI	-	-
62	**Pentacosane**	***n*-alkane**	**2499**	**2500**	**352**	**267, 281, 295, 309, 323**	**MS, LRI**	**A, Al, P**	**A, Al, K, P**
63	**Unidentified ketone 2 [b]**	**Ketone**	**2509**	**-**	**-**	**58, 59, 71, 85, 96, 239**	**MS**	**-**	**-**
64	**7-methylpentacosane †**	**Methyl alkane**	**2522**	**2530**	**362**	**113, 127, 141, 155, 169, 183, 197, 224, 239, 253, 267, 281, 295, 309**	**MS, LRI**	**P**	**-**
65	**11-methylpentacosane †**	**Methyl alkane**	**2530**	**2530**	**366**	**168, 169, 196, 224, 225**	**MS, LRI**	**P**	**P**
66	**13-methylpentacosane †**	**Methyl alkane**	**2569**	**2530**	**366**	**127, 139, 141, 169, 183, 197, 225, 229, 254**	**MS, LRI**	**P**	**P**
67	**3-methylpentacosane**	**Methyl alkane**	**2574**	**2574**	**366**	**337, 336, 253, 267, 281, 309**	**MS, LRI**	**P**	**Al, P**
68	**11,15-dimethylpentacosane †**	**Dimethyl alkane**	**2584**	**2550**	**380**	**168, 169, 239**	**MS, LRI**	**P**	**P**
69	**Hexacosane**	***n*-alkane**	**2600**	**2600**	**366**	**281, 295, 309, 323, 337, 351**	**MS, LRI**	**P**	**A, Al, P**
70	Tetracosanal	Aldehyde	2637	2632	352	334, 306, 278, 264, 250	MS, LRI	P	P
71	**2-methylhexacosane**	**Methyl alkane**	**2663**	**2664**	**380**	**280, 337, 364, 365**	**MS, LRI**	**P**	**P**
72	**Unidentified hydrocarbon 8**	**-**	**2672**	**-**	**-**	**337, 211, 225, 253, 351**	**MS**	**-**	**-**
73	**13-methylhexacosane †**	**Methyl alkane**	**2682**	**2633**	**380**	**196, 197, 308, 309, 211, 280**	**MS, LRI**	**P**	**P**
74	**Unidentified hydrocarbon 9**	**-**	**2690**	**-**	**-**	**99, 97, 113, 127, 169, 225, 280**	**MS**	**-**	**-**
75	**Heptacosane**	***n*-alkane**	**2704**	**2700**	**380**	**323, 337, 351, 365**	**MS, LRI**	**P**	**Al, P**
76	**7-methylheptacosane †**	**Methyl alkane**	**2712**	**2730**	**394**	**99, 97, 113, 127, 141, 155, (224), 225, 309, 337**	**MS, LRI**	**P**	**-**
77	Unidentified ketone 3	Ketone	2723	-	-	59, 58, 96, 111, 125, 137, 250	MS	-	-
78	**13-methylheptacosane**	**Methyl alkane**	**2737**	**2733**	**394**	**168, 196, 197, 224, 253**	**MS, LRI**	**P**	**P**
79	**Unidentified hydrocarbon 10**	**-**	**2755**	**-**	**-**	**267, 295, 195, 197, 224**	**MS**	**-**	**-**
80	**11-methylheptacosane †**	**Methyl alkane**	**2759**	**2734**	**394**	**127, 141, 155, 168, 169, 239, 252, 253**	**MS, LRI**	**P**	**P**
81	**2-methylheptacosane**	**Methyl alkane**	**2764**	**2760**	**394**	**141, 183, 351**	**MS, LRI**	**P**	**P**
82	Docosyl pentyl ether	Ether	2770	2775	396	71, 83, 97, 111, 125, 139, 153, 167	MS, LRI	-	-
83	**3-methylheptacosane**	**Methyl alkane**	**2774**	**2773**	**394**	**365, 267, 281, 295, 309, 337**	**MS, LRI**	**P**	**P**
84	**5,15- or 5,17-dimethylheptacosane †**	**Dimethyl alkane**	**2777**	**2778**	**408**	**168, 127, 155, 211, 239**	**MS, LRI**	**-**	**-**
85	**5,11-dimethylheptacosane †**	**Dimethyl alkane**	**2784**	**2783**	**408**	**99, 113, 127, 141, 155, 168, 169, 239**	**MS, LRI**	**-**	**P**
86	**Octacosane**	***n*-alkane**	**2800**	**2800**	**394**	**337, 351, 365, 379**	**MS, LRI**	**A, P**	**A, P**

Table 1. *Cont.*

No.	Compound	Class	LRI Rxi-5Sil	Lit. LRI	M+ (*m*/*z*)	Other Confirmatory MS Peaks (*m*/*z*)	Ident. ^	Roles # (Coleoptera)	Roles # (Other Insects)
87	Squalene	Triterpenoid	2811	2790	410	69, 81, 95, 121, 136, 137, 149	MS, LRI	P	A, P
88	**12-methyloctacosane †**	**Methyl alkane**	**2830**	**2829**	**408**	**224, 210, 211, 182, 183, 197**	**MS, LRI**	**P**	**P**
89	Hexacosanal	Aldehyde	2837	2834	380	57, 71, 82, 96, 111, 124, 180, 362	MS, LRI	-	P
90	**x-methyloctacosane †**	**Methyl alkane**	**2858**	**2864**	**408**	**365, 253, 281, 295**	**MS, LRI**	**-**	**-**
91	**2-methyloctacosane**	**Methyl alkane**	**2865**	**2864**	**408**	**365, 253, 267, 281, 295, 309**	**MS, LRI**	**P**	**P**
92	**Nonacosene**	**Alkene**	**2881**	**2888**	**406**	**97, 83, 125, 167, 195**	**MS, LRI**	**-**	**-**
93	1-hexacosanol	Fatty alcohol	2890	2865	382	57, 97, 83, 69, 71, 111, 125, 153, 167, 181, 195, 209	MS, LRI	-	P
94	**Nonacosane**	***n*-alkane**	**2918**	**2900**	**408**	**337, 351, 365, 379, 393**	**MS, LRI**	**P**	**A, Al, P**
95	**Triacontane**	***n*-alkane**	**2982**	**3000**	**422**	**168, 169, 224, 197**	**MS, LRI**	**P**	**A, P**
96	**x,12-dimethylnonacosane †**	**Dimethyl alkane**	**3002**	**3000**	**437**	**112, 113, 169, 182, 183, 336, 337**	**MS, LRI**	**-**	**-**
97	**2-methyltriacontane †**	**Methyl alkane**	**3039**	**3058**	**437**	**239, 224, 337, 365**	**MS, LRI**	**P**	**P**

^ Identification methods: LRI = linear retention index; MS = mass spectra, # identified roles from El-Sayed [22]: A = attractant; Al = allomone; K = kairomone; P = pheromone, † tentative identification, a "Undetermined B" from Souza et al. [16], b may be "Undetermined G" from Souza et al. [16].

Table 2. Non-CHC volatile compounds identified in the Gonipterini hexane extracts by using GC-MS. Compounds were quantified as relative percentages of the total peak areas in the total ion chromatogram (TIC).

No.	Compound	*B. squamicollis* (n = 3)	*G. cinnamomeus* (n = 3)	*G.* sp. n. 2 (n = 1)	*O. fasciculatus* (n = 5)	*Oxyops* sp. 1 (n = 3)	*p* Value
1	3-hexanone	0.03 ± 0.01	0.06 ± 0.01	0.08	0.05 ± 0.04	0.04 ± 0.00	NS
2	2-hexanone	0.02 ± 0.02	0.07 ± 0.01	0.07	0.06 ± 0.04	0.04 ± 0.01	NS
4	Heptanal	0.03 ± 0.01	0.03 ± 0.02	0	0	0	*
5	Octanal	0.01 ± 0.01	0	0	0	0	NS
6	Eucalyptol	0	0	0.10	0.01 ± 0.02	0	***
9	Nonanal	0.13 ± 0.05	0	0	0	0	***
10	Decanal	0.01 ± 0.01	0.02 ± 0.04	0	0	0	NS
11	Exo-2-hydroxycineole	0.04 ± 0.02	0.08 ± 0.04	0	0.02 ± 0.02	0	*
13	10-undecenal	0.14 ± 0.17	0	0	0	0	NS
14	Tentative: carvacrol	2.42 ± 3.83	0.04 ± 0.07	0	0.02 ± 0.04	0	NS
15	Tentative: isoascaridole	0.01 ± 0.01	0	0	0	0	NS
16	Tentative: 4a-methyldecahydro-1-naphthalenol	0.21 ± 0.23	0	0	0	0	NS
18	Tentative: cis-p-menth-1-en-3,8-diol	0.02 ± 0.03	0	0	0	0	NS
19	(+)-cis,trans-nepetalactone	0.02 ± 0.03	0	0	0	0	NS
20	Dodecanal	0	0.02 ± 0.03	0	0.01 ± 0.01	0.01 ± 0.02	NS
21	Aromadendrene	0	0	0	0.01 ± 0.03	0	NS
23	Bicyclogermacrene	0.03 ± 0.05	0	0	0.02 ± 0.02	0	NS
25	Globulol	0	0	0.07	0.01 ± 0.03	0	NS
26	Tetradecanal	0.04 ± 0.02	0	0.05	0.01 ± 0.02	0.02 ± 0.03	NS

Table 2. *Cont.*

No.	Compound	*B. squamicollis* (n = 3)	*G. cinnamomeus* (n = 3)	*G.* sp. n. 2 (n = 1)	*O. fasciculatus* (n = 5)	*Oxyops* sp. 1 (n = 3)	*p* Value
29	*cis*-9-hexadecenal	0.05 ± 0.01	0	0	0	0	***
30	Hexadecanal	0.17 ± 0.05	0.09 ± 0.04	0.10	0.16 ± 0.13	0.13 ± 0.08	NS
31	6,10,14-trimethyl-2-pentadecanone	0	0	0	0	0.01 ± 0.02	NS
32	2-heptadecanone	0.09 ± 0.02	0.07 ± 0.03	0.14	0.04 ± 0.05	0.06 ± 0.02	NS
36	9-octadecanone	0	0	0	0.1 ± 0.1	0	NS
37	*cis*-13-octadecenal	0.12 ± 0.03	0	0	0	0	***
38	Tentative: *cis*-9-octadecenal	0	0	0	0.03 ± 0.07	0	NS
39	Octadecanal	0.61 ± 0.14	0.07 ± 0.04	0.13	0.19 ± 0.18	0.16 ± 0.13	**
40	*cis*-2-octadecen-1-ol acetate	0	0	0	0.01 ± 0.01	0	NS
41	2-nonadecanone	0.09 ± 0.02	0.04 ± 0.04	0.18	0.02 ± 0.05	0.08 ± 0.05	NS
42	Nonadecanal	0.06 ± 0.01	0	0	0	0	***
49	Eicosanal	0.09 ± 0.04	0	0.71	0.03 ± 0.07	0	***
52	Unidentified ketone 1	0	0	0.09	0	0	***
53	Henicosanal	0	0	0.50	0	0	***
55	Unidentified aldehyde	0.02 ± 0.01	0	0	0	0	*
58	Docosanal	0.01 ± 0.01	0.58 ± 0.31	1.12	0	0	***
63	Unidentified ketone 2	0	0.05 ± 0.09	0.13	0	0	NS
70	Tetracosanal	1.95 ± 2.56	4.22 ± 0.91	0	0	0.16 ± 0.27	**
77	Unidentified ketone 3	0	0.75 ± 0.31	0	0	0	***
82	Docosyl pentyl ether	0	0	0	0	0.27 ± 0.47	NS
87	Squalene	0.38 ± 0.65	0.36 ± 0.37	0.71	0.43 ± 0.20	0.27 ± 0.08	NS
89	Hexacosanal	0.94 ± 0.24	0.94 ± 0.3	0	0	0	***

NS = not significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

A total of 56 of the volatile compounds had some pheromone-type activity in one or more insect species (Table 2), with 43 being documented as having pheromone-type activity in Coleoptera [22]. Several of the compounds (aromadendrene and exo-2-hydroxycineole) have been previously identified as attractants for *Gonipterus platensis* [25]. Eucalyptol (1,8-cineole) is reportedly used as a defensive agent by *Oxyops vitiosa* larvae [26], in addition to acting as a potential attractant in adults of this species [27]. A number of 1,8-cineole metabolites have also been identified as pheromones in *Gonipterus platensis* [28]. No previous work was found on attractants for *Bryachus*.

Table 2 details the concentrations of the non-CHC volatile compounds found in each of the five Gonipterini species, while Table 3 compares the CHC contents among the species. The most abundant CHCs across all five species were nonacosane and 7-methylheptacosane. *B. squamicollis* also contained high levels of heptacosane, while both *Gonipterus* species showed high levels of hexacosane. *Oxyops* sp. 1 notably contained quite high concentrations (8.28 ± 6.05%) of 2-methyloctacosane, as well as a lower 7-methylheptacosane concentration than that of any other species.

Table 3. Cuticular hydrocarbons (CHCs) identified in the Gonipterini hexane extracts by using GC-MS. Compounds were quantified as relative percentages of the total peak areas in the total ion chromatogram (TIC).

No.	Compound	*B. squamicollis* (n = 3)	*G. cinnamomeus* (n = 3)	*G.* sp. n. 2 (n = 1)	*O. fasciculatus* (n = 5)	*Oxyops* sp. 1 (n = 3)	*p* Value
3	2,4-dimethylheptane	0.02 ± 0.01	0.02 ± 0.02	0.05	0.03 ± 0.02	0.02 ± 0.00	NS
7	3,6-dimethyldecane	0.03 ± 0.01	0.06 ± 0.02	0.06	0.05 ± 0.03	0.03 ± 0.01	NS
8	2,6,8-trimethyldecane	0.02 ± 0.02	0.02 ± 0.03	0	0.01 ± 0.03	0.01 ± 0.01	NS
12	Tentative: 2,6,10-trimethylundecane	0.03 ± 0.01	0.06 ± 0.01	0.05	0.06 ± 0.03	0.03 ± 0.00	NS
17	4,6-dimethyldodecane	0.03 ± 0.01	0.02 ± 0.03	0	0.03 ± 0.03	0.01 ± 0.02	NS
22	Unidentified hydrocarbon 1	0.01 ± 0.01	0.01 ± 0.02	0.06	0.05 ± 0.02	0.03 ± 0.00	NS
24	Tentative: 2,6,10-trimethyltridecane	0.01 ± 0.01	0.01 ± 0.02	0	0	0	NS
27	Heptadecane	0.03 ± 0.01	0.05 ± 0.01	0.05	0.04 ± 0.03	0.02 ± 0.02	NS
28	Phytane	0.01 ± 0.02	0	0	0	0	NS
33	Tentative: 2,2-dimethyloctadecane	0.04 ± 0.02	0.06 ± 0.06	0.09	0.03 ± 0.03	0.02 ± 0.03	NS
34	Heptadecanal	0.05 ± 0.01	0	0	0	0	***
35	Tentative: 3-ethyl-3-methylheptadecane	0.03 ± 0.02	0.06 ± 0.02	0.05	0.06 ± 0.03	0.03 ± 0.00	NS
43	Unidentified hydrocarbon 2	0.23 ± 0.21	0.25 ± 0.43	0	0.03 ± 0.07	0.12 ± 0.22	NS
44	Unidentified hydrocarbon 3	0	0.35 ± 0.60	0	0	0.23 ± 0.40	NS
45	Unidentified hydrocarbon 4	0.48 ± 0.46	0.42 ± 0.73	0	0.06 ± 0.14	0.28 ± 0.48	NS
46	Unidentified hydrocarbon 5	0.10 ± 0.08	0.14 ± 0.24	0.07	0.04 ± 0.02	0.01 ± 0.02	NS
47	Unidentified hydrocarbon 6	0.08 ± 0.08	0.09 ± 0.15	0	0	0.28 ± 0.49	NS
48	Docosane	0.02 ± 0.02	0	0	0.06 ± 0.05	0	NS
50	Unidentified hydrocarbon 7	0	0	0	0	0.03 ± 0.03	*
51	Tricosane	0.74 ± 0.19	0.37 ± 0.09	0	1.35 ± 0.54	0.27 ± 0.06	**
54	11-methyltricosane	0.20 ± 0.34	0	0	0	0.02 ± 0.04	NS
56	3-methyltricosane	0.02 ± 0.03	0	0	0	0	NS
57	Tetracosane	0.03 ± 0.02	0.07 ± 0.01	0.16	0.09 ± 0.05	0.02 ± 0.02	*
59	Tentative: 9-methyltetracosane	0.01 ± 0.02	0	0	0	0	NS
60	2-methyltetracosane	0	0.94 ± 1.64	0	0	0.69 ± 1.19	NS
61	Tentative: x-pentacosene	0	0	0	0.03 ± 0.07	0	NS
62	Pentacosane	1.67 ± 0.93	1.83 ± 0.37	1.82	4.26 ± 2.02	0.97 ± 0.88	NS
64	Tentative: 7-methylpentacosane	0	0.27 ± 0.46	0	0.48 ± 0.42	0	NS
65	Tentative: 11-methylpentacosane	0	0.15 ± 0.26	0	0.11 ± 0.14	0	NS
66	Tentative: 13-methylpentacosane	0.03 ± 0.05	0	0	0	0	NS
67	3-methylpentacosane	0.26 ± 0.23	1.09 ± 0.64	0.27	0	0	**
68	Tentative: 11,15-dimethylpentacosane	0	0	0	0.70 ± 1.57	0	NS
69	Hexacosane	9.04 ± 6.87	13.26 ± 7.6	14.70	8.30 ± 7.10	11.01 ± 8.76	NS
71	2-methylhexacosane	0	0	0	0.03 ± 0.07	0	NS
72	Unidentified hydrocarbon 8	0	0.51 ± 0.46	0	0	0.15 ± 0.15	NS
73	Tentative: 13-methylhexacosane	0.65 ± 1.12	0.02 ± 0.04	0	0.88 ± 1.37	0	NS
74	Unidentified hydrocarbon 9	0.36 ± 0.62	0	0	0	0	NS
75	Heptacosane	11.32 ± 2.95	4.3 ± 4.94	2.27	1.54 ± 3.44	0.36 ± 0.62	*
76	Tentative: 7-methylheptacosane	21.49 ± 5.33	28.94 ± 7.24	25.45	27.58 ± 6.29	16.77 ± 6.68	NS
78	13-methylheptacosane	0.32 ± 0.06	0.07 ± 0.13	0	0.08 ± 0.15	0.73 ± 0.24	**
79	Unidentified hydrocarbon 10	1.14 ± 1.2	0	0	0.01 ± 0.02	0.10 ± 0.17	NS
80	Tentative: 11-methylheptacosane	0	4.60 ± 4.16	0	1.18 ± 2.49	0.14 ± 0.23	NS
81	2-methylheptacosane	0	0	0	0	0.08 ± 0.07	*
83	3-methylheptacosane	0	3.14 ± 1.61	2.82	0	0	***
84	Tentative: 5,15- or 5,17-dimethylheptacosane	1.06 ± 1.07	0	0	0	0	NS
85	Tentative: 5,11-dimethylheptacosane	0.36 ± 0.62	1.93 ± 3.35	0	3.93 ± 7.21	0.79 ± 1.37	NS
86	Octacosane	6.89 ± 2.27	3.55 ± 0.89	19.10	4.16 ± 0.85	4.43 ± 1.34	***

Table 3. *Cont.*

No.	Compound	*B. squamicollis* (n = 3)	*G. cinnamomeus* (n = 3)	*G.* sp. n. 2 (n = 1)	*O. fasciculatus* (n = 5)	*Oxyops* sp. 1 (n = 3)	*p* Value
88	Tentative: 12-methyloctacosane	0.22 ± 0.14	0.05 ± 0.08	0	0	0	*
90	Tentative: x-methyloctacosane	0.05 ± 0.05	0	0.11	0	0	***
91	2-methyloctacosane	0.24 ± 0.14	0.93 ± 0.22	0.96	0	8.28 ± 6.05	**
92	Nonacosene	1.03 ± 0.40	0	0	0	0	*
93	1-hexacosanol	0	0.21 ± 0.20	0	0	0	***
94	Nonacosane	26.54 ± 5.05	17.34 ± 3.08	24.90	42.99 ± 14.09	51.41 ± 13.58	*
95	Triacontane	5.72 ± 0.63	7.31 ± 3.07	2.79	0.51 ± 0.37	1.38 ± 0.68	***
96	Tentative: x,12-dimethylnonacosane	0.30 ± 0.50	0	0	0.01 ± 0.02	0	NS
97	Tentative: 2-methyltriacontane	1.38 ± 0.43	0	0	0	0	***

NS = not significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The most abundant compound class was methyl alkanes (with a total of 18 compounds present), followed by aldehydes (16), *n*-alkanes (10), ketones (8), and dimethyl alkanes (8) (Table 2). As shown in Table 4, the greatest number of total compounds were found in *B. squamicollis* (71), and the greatest number of unique compounds was found only in this species (20, comprising 28.2% of the total volatile compounds found in this species). *Gonipterus* sp. n. 2 contained the lowest number of compounds (35), in addition to possessing only two unique compounds (henicosanal and an unidentified ketone). A total of 23 compounds were identified as being present across all five species.

Table 4. Summary of the numbers of compounds identified in each Gonipterini species.

Category	*B. squamicollis*	*G. cinnamomeus*	*G.* sp. n. 2	*O. fasciculatus*	*Oxyops* sp. 1
Number of identified compounds	71	54	35	52	45
Number of unique compounds	20	2	2	7	4
Percentage of unique compounds	28.2%	3.7%	5.7%	13.5%	8.9%
Number of identified CHCs	43	37	20	34	33
Number of unique CHCs	8	1	0	3	2
Percent of unique CHCs	18.6%	2.7%	0%	8.8%	6.1%

3.2. Chemometric Analysis

To investigate the natural groupings in the CHC data, an unsupervised exploratory analysis was conducted on the CHC data only. Prior to the analysis, the volatile data were subjected to a centred log-ratio (clr) transformation, as recommended by Brückner and Heethoff [29] for similar datasets.

The principal component analysis (PCA) revealed a broad separation between *B. squamicollis* and the remaining species across the first principal component (PC 1), which explained 18.7% of the variation in the CHC dataset. The remaining species were largely separated across PC 2, which explained a further 12.9% of the variation (Figure 2). Most species were well separated across the first two PCs, although the single specimen of *Gonipterus* sp. n. 2 was quite close to the *Oxyops* sp. 1 cluster.

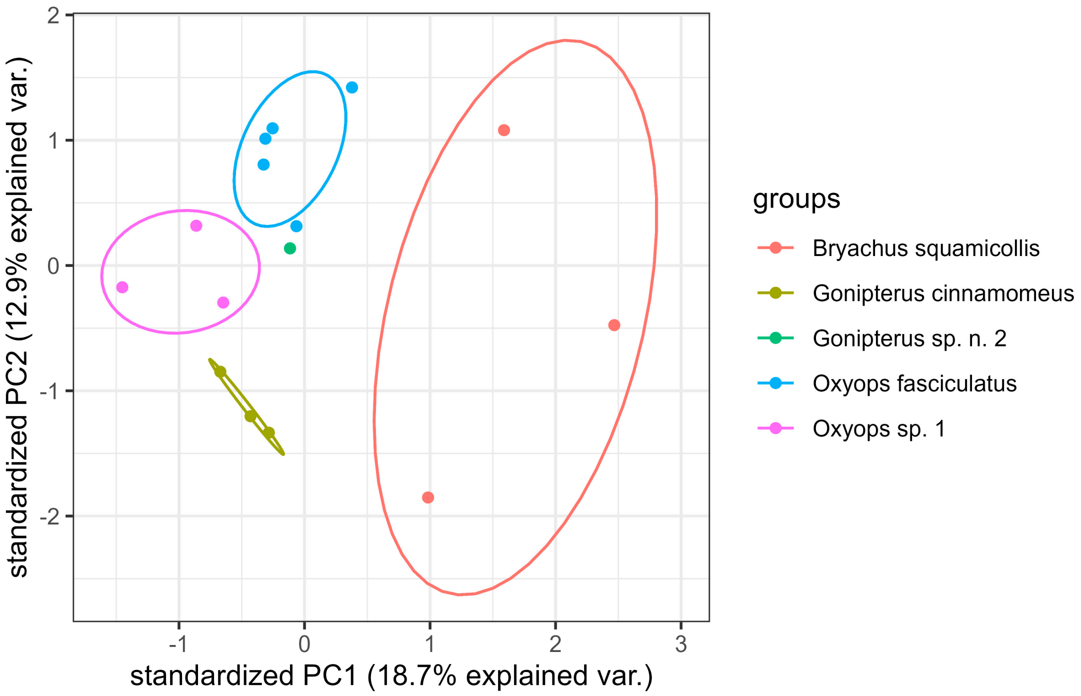

Figure 2. Score plot showing the results of the principal component analysis performed on the clr-transformed CHC data.

Examination of the PCA loadings plot (Figure 3) was used to investigate the compounds that were most strongly associated with particular Gonipterini species. For example, nonacosane was strongly associated with *Oxyops* sp. 1, while the large number of compounds loaded in the same direction as *B. squamicollis* supported previous observations about the large number of unique compounds found in this species (Table 3).

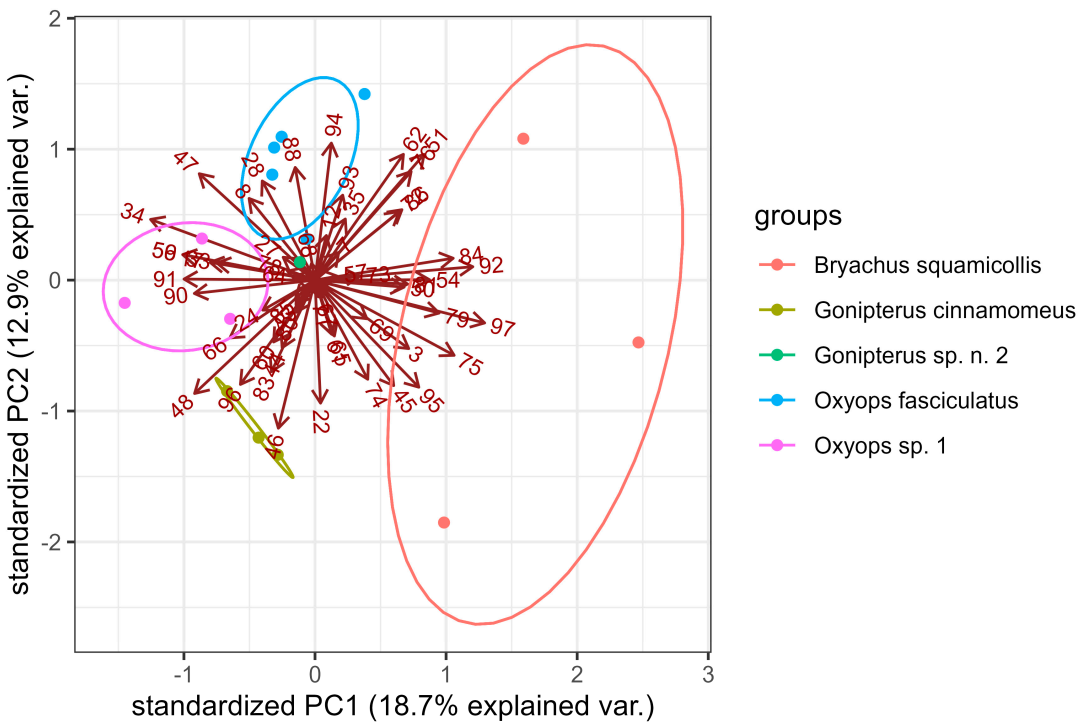

Figure 3. PCA loadings plot showing the influence of individual CHCs on the principal component analysis performed on the clr-transformed CHC data.

4. Discussion

Souza et al. [16] previously reported the CHC profile of *Gonipterus* sp. n. 2, with the major compounds present including *n*-heptacosane, 2-methylhexacosane, *n*-hexacosane, *n*-pentacosane, and *n*-octacosane. Somewhat contrasting results were found in this study, with the major compounds from this species being identified as 7-methylheptacosane, nonacosane, octacosane, hexacosane, 3-methylheptacosane, triacontane, heptacosane, and pentacosane. However, it should be noted that only one specimen from this species was analysed, so the results here may not necessarily be representative of the species as a whole. Another potential reason may be the difference in geographic locations. The present study used a specimen from central Queensland, while Souza et al. [16] collected Gonipterini specimens from a much wider region across Australia (Qld, NSW, ACT, Vic, WA). Studies have shown that CHC profiles can vary significantly with geographic location [16,30,31]. Finally, the species is part of a cryptic complex [17], so there is the possibility of misidentification, as genetic analysis was not performed in this study.

The major CHCs from *G. cinnamomeus* were found to be 7-methylheptacosane, nonacosane, hexacosane, and triacontane in this study, quite similarly to *Gonipterus* sp. n. 2. The CHC profile of this species does not appear to have been previously reported.

Souza et al. [16] also studied the CHC profiles of ten *Oxyops* specimens (not identified to species), reporting the major constituents as *n*-heptacosane, *n*-pentacosane, two unidentified compounds, and *n*-nonacosane. This largely concurred with the predominant CHCs found from *O. fasciculatus* in this study: nonacosane, 7-methylheptacosane, hexacosane, pentacosane, and octacosane. The CHC profile of *Oxyops* sp. 1 was somewhat less similar to the general *Oxyops* profile reported by Souza et al. [16]. The major constituents included nonacosane, 7-methylheptacosane, and hexacosane; however, it was unique in having a particularly high concentration of 2-methyloctacosane (8.28%) and the lowest concentration of pentacosane (0.97%) out of all species studied. This species (*Oxyops* sp. 1) has not yet been formally described yet; hence, its status in the *Oxyops* genus remains to be confirmed by a thorough morphological investigation and genetic study.

The results of the PCA supported *B. squamicollis* as the outgroup taxon. Within the remaining species, the *Oxyops* and *Gonipterus* species were loosely clustered together, but with some overlap.

Although CHC composition is primarily regulated through genetic means [9], it can be impacted by a range of factors, including diet [32,33], population age structure [34,35], locality, and climate [36,37]. However, a number of studies have found that CHC profiles are reasonably stable among different locations and ecological factors [16,38,39]. Furthermore, any impact of most of these variables would be expected to be minimal in this study, given that all specimens were collected on the same day from the same vicinity and were all collected from the same host plant species (*E. populnea*).

The overall results of this work support the prospect of using CHC profiles as a (relatively) rapid method of discriminating between Gonipterini genera and species. Such an approach has previously been applied across a range of insect orders to date, although the bulk of studies have been performed on Hymenoptera or Diptera [40–42]. CHC profiling shows particular promise when combined with other taxonomic techniques, including DNA barcoding and morphological analysis [40,43,44]. Such rapid analytical tools for discriminating between Gonipterini species could find use in a variety of applications, including identifying large numbers of specimens from field surveys or supporting the description of new species alongside DNA barcoding or morphological studies.

5. Conclusions

This study presented the cuticular hydrocarbon profiles of several Gonipterini species for the first time, including *Bryachus squamicollis*, *Gonipterus cinnamomeus,* and *Oxyops fasciculatus*. Principal component analysis revealed broadly differing CHC profiles between most species investigated, with *B. squamicollis* demonstrating the greatest divergence from the other Gonipterini genera/species. The results suggest that CHC analysis could be used to support established taxonomic methods, including the use of morphological features and genetic sequencing results.

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