




“Quinone Millipedes” Reconsidered: Evidence for a Mosaic-Like Taxonomic Distribution of Phenol-Based Secretions Across the Julidae

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Abstract The defensive chemistry of juliformian millipedes is characterized mainly by benzoquinones (“quinone millipedes”), whereas the secretions of the putative close outgroup Callipodida are considered to be exclusively phenolic. We conducted a chemical screening of julid secretions for phenolic content. Most species from tribes Cylandroiulini (15 species examined), Brachyiulini (5 species examined), Leptoiulini (15 species examined), Uncigerini (2 species examined), Pachyiulini (3 species examined), and Ommatoiulini (2 species examined) had non-phenolic, in most cases exclusively benzoquinonic secretions. In contrast, tribes Cylandroiulini, Brachyiulini, and Leptoiulini also contained representatives with predominantly phenol-based exudates. In detail, *p*-cresol was a major compound in the secretions of the cylandroiulines *Styrioiulus pelidnus* and *S. styricus* (*p*-

cresol content 93 %) and an undetermined *Cylandroiulus* species (*p*-cresol content 51 %), in the brachyiulines *Brachyiulus lusitanus* (*p*-cresol content 21 %) and *Megaphyllum fagorum* (*p*-cresol content 92 %), as well as in an undescribed *Typhloiulus* species (*p*-cresol content 32 %, Leptoiulini). In all species, *p*-cresol was accompanied by small amounts of phenol. The secretion of *M. fagorum* was exclusively phenolic, whereas phenols were accompanied by benzoquinones in all other species. This is the first incidence of clearly phenol-dominated secretions in the Julidae. We hypothesize a shared biosynthetic route to phenols and benzoquinones, with benzoquinones being produced from phenolic precursors. The patchy taxonomic distribution of phenols documented herein supports multiple independent regression events in a common pathway of benzoquinone synthesis rather than multiple independent incidences of phenol biosynthesis.

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Keywords Chemical defense · Quinone millipedes · Phenols · Julida · Julidae · Cylandroiulini · Brachyiulini · Leptoiulini · Diplopoda

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Introduction

Millipedes are well protected against predators. Apart from hardened cuticles, disturbance-related coiling behaviors, or defensive bristles (Polyxenida), the majority of millipedes are known to release repellent and noxious fluids from serial exocrine glands, serving as an effective means of active defense. Depending on the taxonomic group, diplopod defensive secretions show considerable chemical diversity as comprehensively reviewed recently (Shear 2015). Briefly, Glomerida and Polyzoniida produce unique alkaloids (e.g., Meinwald et al. 1966, 1975; Schildknecht et al. 1966; Shear et al. 2011; Wood et al. 2000), Polydesmida produce cyanogenic

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compounds (Makarov et al. 2011; Shear et al. 2007), whereas all groups of Juliformia mainly secrete quinones (e.g., Eisner et al. 1978; Vujisić et al. 2011). Indeed, the latter group - the Juliformia - have been known as “quinone millipedes” (Eisner et al. 1978), implying that this superorder almost exclusively produces quinone-based secretions. Interestingly, within the putative juliformian sister group “Polydesmida (Chordeumatida (Stemmiulida + Callipodida))” several taxa rely on phenolic secretions (e.g., Blanke and Wesener 2014; Shear 2015). Callipodids, for instance, appear to exclusively produce phenols (e.g., Makarov et al. 2011), which give them a characteristic and obtrusive odor, easily noticeable by humans over distances of several meters. Additionally, stemmiulidans (Shear 2015) and several polydesmidans have been reported to discharge phenolics, and in both cases the most frequently occurring component was *p*-cresol (Duffey et al. 1977; Mori et al. 1994; Noguchi et al. 1997; Shear et al. 2007; Taira et al. 2003).

In many Arthropoda, the biosynthesis of phenols and benzoquinones is thought to be related. There are good examples of arthropods that utilize both phenolic and benzoquinonic compounds (e.g., Rocha et al. 2013). In laniatorean harvestmen, for instance, the secretions of representatives of basal grassatorean families rely on phenolics, whereas the secretions of derived grassatoreans may contain both phenols and benzoquinones or may even be purely benzoquinonic (Föttinger et al. 2010; Raspotnig et al. 2015). In laniatoreans, benzoquinones appear to present a derived character state, that can be produced from phenols by para-oxidation, and the production of benzoquinones may be considered an extension of the pathway to phenols (Raspotnig et al. 2015; Rocha et al. 2013).

In the scope of our studies on the evolutionary history of secretion chemistry in diplopods, it is essential to pinpoint basic correlations between the chemistry of different taxa. We here hypothesize that benzoquinones in juliformians evolved from the ancestral state of phenolic secretions, as still present in juliformian outgroups (Raspotnig and Bodner 2014). To test our idea, we conducted extensive chemical screening of julid secretions for their phenolic content. So far, phenolics in juliformians have been regarded to be an exception or even completely missing: only one representative of Julida, namely a member of the basal julidan family Parajulidae, *Oriulus venustus* (Wood 1864), was reported to discharge a phenol-rich secretion (Kluge and Eisner 1971). There are, however, an increasing number of reports on julid phenolics as minor, trace, or by-products of the quinone-rich secretions of certain species (e.g., Sekulić et al. 2014).

We provide evidence that phenol-based secretions are more widespread in the Julidae, and that phenolics even prevail over quinones in some species of the tribes *Cylindroiulini*, *Brachyiulini*, and *Leptoiulini*.

Methods and Materials

Collection of Species Individuals, mostly adults, from 42 species belonging to 17 genera and 6 tribes of Julidae and 2 species of Blaniulidae (see supplemental Table S1) were collected by hand, either from the leaf litter layer or from deeper soil and breakstone beneath the leaf litter layer at various locations in Austria, Germany, Italy, France, Spain, Bulgaria, Serbia, and Azerbaijan. Vouchers were deposited at the Natural History Museum of Vienna (NHMW), Natural History Museum of Denmark (ZMUC), Hungarian Natural History Museum (HNHM), National Museum of Natural History Sofia (NMNHS), and at the Faculty of Biology, Institute of Zoology, University of Belgrade (FBIZO).

Extraction and Analysis of Defensive Secretions Secretions were obtained by whole body extraction of single individuals in hexane for 15 min. The defensive secretions were discharged directly into the solvent. Aliquots of diluted extracts (1.5 μl) were analyzed by gas chromatography– mass spectrometry (GC-MS), using a trace gas chromatograph coupled to a DSQ I mass spectrometer (MS), both from Thermo (Vienna, Austria). GC and MS conditions were the same as previously described (Bodner and Raspotnig 2012). Gas chromatographic retention indices (RI) of extract components were calculated using an alkane standard mixture (Van den Dool and Kratz 1963).

High-resolution mass spectrometry was carried out on a Q-exactive high-resolution Orbitrap MS from Thermo (Vienna, Austria). Samples containing secretion in hexane were gently reduced under nitrogen, then dissolved in methanol: water (1:1) with 1 % formic acid, and analyzed by direct infusion ESI-MS and HPLC-MS, respectively. Components were observed as $[M + H]^+$ - ions as well as Na - and K - adducts.

Reference Compounds and Derivatization For comparison of GC-MS data to authentic reference compounds 1,4-benzoquinone, 2,3-dimethoxy-5-methyl-1,4-benzoquinone, *p*-cresol, *o*-cresol, and *m*-cresol, methyl-paraben, and 2-phenylphenol were purchased from Sigma (Vienna, Austria). 2,3,5,6-Tetramethoxy-1,4-benzoquinone was from MicroCombiChem (Wiesbaden, Germany), and 2,3-dimethoxy-5-methyl-1,4-hydroquinone from abcr GmbH & Co KG (Karlsruhe, Germany). As reference for other compounds, particularly 2-methyl-1,4-benzoquinone, 2-hydroxy-3-methyl-1,4-benzoquinone, 2-methoxy-3-methyl-1,4-benzoquinone, 2,3-dimethoxy-1,4-benzoquinone, 2-methylhydroquinone, we used natural sources from which these compounds had already been identified (*Allajulus dicentrus*: Bodner and Raspotnig 2012; *Cylindroiulus boleti*: Vujisić et al. 2011).

Table 1 Chemical classes in the defensive secretions of Julida

family	species		phenols	quinones	other		
Blaniulidae	<i>Blaniulus</i>	<i>dolfusi</i>	–	+	+		
		<i>guttulatus</i> *	–	+	+		
		<i>Cibiniulus phlepsi</i> *	–	+	+		
		<i>Nopoiulus kochii</i> *	–	+	+		
Julidae	Brachyiulini	<i>Proteroiulus fuscus</i>	–	+	+		
		<i>Anaulaciulus okinawaensis</i> *	–	+	–		
		<i>sp.</i> *	–	+	+		
		<i>Brachyiulus lusitanus</i>	+	+	+		
	Cylindroiulini	<i>Megaphyllum</i>	<i>bosniense</i> *	–	+	–	
			<i>fagorum</i>	+	–	–	
			<i>hercules</i>	–	+	+	
			<i>silvaticus</i>	–	+	+	
		Cylindroiulini	<i>Allaiulus</i>	<i>unilineatum</i> *	–	+	–
				<i>dicentrus</i> *	–	+	+
				<i>molybdinus</i>	–	+	+
				<i>nitidus</i> *	–	+	–
			<i>Cylindroiulus</i>	<i>apenninorum</i>	–	+	+
				<i>boleti</i> *	–	+	–
				<i>broti</i>	–	+	+
<i>caeruleocinctus</i> *	–			+	+		
<i>londinensis</i> *	–			+	–		
<i>luridus</i> *	–			+	–		
<i>meinerti</i> *	–			+	+		
<i>Enantiulus</i>	<i>sp. (present study)</i>	+	+	–			
	<i>karawankianus</i>	–	+	+			
	<i>nanus</i> *	–	+	+			
<i>Kryphioiulus</i>	<i>transsilvanicus</i>	–	+	+			
	<i>occultus</i>	–	+	+			
<i>Styrioiulus</i>	<i>pelidnus</i>	+	+	–			
	<i>styricus</i>	+	+	–			
Iulini	<i>Julus</i>	<i>scandinavicus</i> *	–	+	+		
Leptoiulini	<i>Lamellotyphlus</i>	<i>sotirovi</i>	–	+	?		
		<i>Leptoiulus proximus</i> *	–	+	+		
		<i>trilineatus</i> *	–	+	–		
	<i>Ophiulus</i>	<i>pilosus</i> *	–	+	–		
		<i>Typhloiulus</i>	<i>spec.nov.</i>	+	+	+	
	<i>bureschi</i>		–	+	+		
	<i>georgievi</i>		–	+	+		
	<i>lobifer</i>		–	+	?		
	<i>nevoi</i>		–	+	?		
	<i>orpheus</i>		–	+	+		
	<i>serborum</i>		+	+	?		
	<i>Serboiulus</i>		<i>deelemani</i>	+	+	?	
			<i>kresnik</i>	–	+	?	
		<i>lucifugus</i>	–	+	?		
	Uncigerini	<i>Xestoiulus</i>	<i>imbecillus</i>	–	+	+	
<i>Unciger foetidus</i> *			–	+	–		
		<i>transsilvanicus</i> *	+	+	+		

Table 1 (continued)

family	species		phenols	quinones	other
Pachyiulini	<i>Dolichoius</i>	<i>hercules</i>	–	+	+
	<i>Pachyiulus</i>	<i>cattarensis</i>	–	+	+
		<i>hungaricus</i> *	–	+	+
Ommatoiulini	<i>Ommatoiulus</i>	<i>bipartitus</i>	–	+	+
		<i>sabulosus</i> *	–	+	+
	<i>Tachypodoiulus</i>	<i>niger</i> *	–	+	+
Parajulidae	<i>Oriulus</i>	<i>delus</i> *	+	+	–
	<i>Uroblaniulus</i>	<i>canadensis</i> *	–	+	–

Only species in bol were investigated in this study. For species marked with * literature data are available (see Shear 2015; Vujisić et al. 2011, 2014). Species in bold and marked with * were reinvestigated

Derivatization of cresol isomers to their corresponding trimethylsilyl (TMS)-ethers was conducted by adding 75 μ l N-methyl-N-(trimethylsilyl)-trifluoroacetamid (MSTFA in pyridine 2:1; with 1 % trimethylchlorosilane [TMCS]) to 50 μ l of secretion in hexane. After 30 min of reaction at 55 °C, an aliquot of the mixture (1.5 μ l) was injected directly into the GC-MS.

Profile Evaluation and Statistics Relative abundance of single components (in %) was calculated by integration of peak areas in the chromatograms, leading to individual secretion profiles. This semi-quantitative method to calculate secretion profiles is widely used in chemosystematic studies of different arthropod taxa (e.g., mites (Heethoff 2012; Sakata et al. 2003); opilionids (Hara et al. 2005); thrips (Suzuki et al. 1989); diplopods (Makarov et al. 2010)). Individual chromatographic profiles were compared eventually by non-metric multidimensional scaling (NMDS) using the Bray-Curtis coefficient of dissimilarity (Bray and Curtis 1957).

Results

Phenol-Producing Julid Species From the 42 species of brachyiuline, cylindroiuline, leptoiuline, uncigerine, pachyiuline, and ommatoiuline Julidae, the secretions of 28 species were investigated for the first time; the same is true for the 2 blaniulids (see Table 1). For the remaining 14 species, literature data from previous investigations were available – these species, however, were re-investigated. Phenol-rich secretions, i.e., phenolic content >20 % of the whole secretion (based on comparison of peak areas), were found for 6 species from 3 different julid tribes: (i) *Brachyiulus lusitanus* and *Megaphyllum fagorum* (both Brachyiulini), (ii) *Styrioiulus styricus*, *S. pelidnus*, and *Cylindroiulus* sp. (Cylindroiulini), and (iii) a yet undescribed species of genus *Typhloiulus* (Leptoiulini).

Compound Identification The major phenolic compound in all 6 species appeared to be a methylated phenol isomer (= cresol: peak D; M^+ at m/z 108). The mass spectra of the three possible cresol isomers (*p*-, *o*-, *m*-cresol) are basically indistinguishable, at best showing slight differences in the intensity of M^+ (at m/z 108) and $M-1^+$ -ions (at m/z 107), respectively. The retention index measured for compound D (RI = 1071) was clearly different from authentic *o*-cresol (RI = 1051), but corresponded to both the RI of authentic *m*-cresol (RI = 1072) as well *p*-cresol (RI = 1071). TMS-Derivatization of compound D, *p*- and *m*-cresol, respectively, led to cresol-TMS ethers, which again showed indistinguishable mass spectra: M^+ at m/z 180 (45), fragment ions at m/z 165 (100), 149 (5), 135 (6), 105 (3), 91 (9). The retention time of the TMS-derivative of compound D, however, corresponded to *p*-cresol-TMS ether only (*m*-cresol-TMS: RI = 1151; *p*-cresol-TMS: RI = 1160; compound D-TMS: RI = 1160). The second phenolic compound of the extracts (peak B: M^+ at m/z 94) was identified as phenol. Quantitatively, *p*-cresol accounted for about 20 % (*B. lusitanus*) up to more than 90 % of the secretions (*Styrioiulus*, *M. fagorum*), whereas phenol generally was a minor or even a trace compound in all 6 species. Only the secretion of *M. fagorum* was purely phenolic.

From the secretions of *B. lusitanus*, *Cylindroiulus* sp., *S. pelidnus*, *S. styricus*, and *Typhloiulus* n. sp. additional 21 components, mainly benzoquinones, could be separated (Table 2, Fig. 1). Fourteen of the compounds were fully or at least partly identified, either by a comparison of GC-MS data to authentic standards, by a comparison of mass spectra and retention indices to data from literature, or by high resolution mass spectrometry, respectively (Table 2). These compounds comprised 1,4-benzoquinone and differentially substituted methyl-, hydroxy-, and methoxy-1,4-benzoquinones (peaks A, C, E, F, G, I, J, L, and M) as well as two hydroquinones (peaks H, M), all of which had been described previously from the secretions of various Juliformia (Shear 2015).

All other compounds (K, N, O, P, Q, R, S, T, U, V, and W) were, to our knowledge, new for millipede secretions.

Table 2 Gas chromatographic and mass spectral data

Peak no.	Retention index RI measured/authentic reference	Mass spectrometric fragmentation <i>m/z</i> (rel. Intensity)	Identified as
A	920	110 ($[M + 2]^+$, 32), 108 (M^+ , 100), 82 (42), 80 (39), 54 (82)	1,4-benzoquinone
B	977	95 ($[M + 1]^+$, 7), 94 (M^+ , 100), 66 (35), 65 (24), 55 (6)	phenol
C	1014	122 (M^+ , 100), 94 (99), 82 (64), 68 (35), 66 (53), 54 (67)	2-methyl-1,4-benzoquinone
D	1071	108 (M^+ , 82), 107 (100), 90 (10), 80 (14), 79 (24), 77 (29)	p-cresol
E	1119	138 (M^+ , 100), 137 (3), 110 (7), 83 (6), 82 (14), 54 (10)	2-hydroxy-3-methyl-1,4-benzoquinone
F	1183	152 (M^+ , 100), 151 (21), 137 (5), 123 (7), 122 (43), 109 (14), 94 (5), 83 (8), 82 (10), 81 (7), 67 (7), 66 (15), 54 (10), 53 (12)	2-methoxy-3-methyl-1,4-benzoquinone
G	1319	170 ($[M + 2]^+$, 16), 168 (M^+ , 100), 155 (7), 153 (39), 140 (1), 138 (6), 125 (13), 123 (88), 122 (13), 112 (2), 95 (11), 94 (5), 82 (10), 69 (25), 54 (12)	2,3-dimethoxy-1,4-benzoquinone
H	1341	124 (M^+ , 100), 123 (42), 107 (8), 105 (4), 95 (15), 77 (6), 69 (7), 67 (10), 57 (10)	2-methylhydroquinone
I	1341	152 (M^+ , 100), 137 (11), 124 (36), 123 (26), 122 (22), 109 (8), 94 (7), 84 (11), 69 (83), 66 (25), 56 (9)	2-methoxy-5-methyl-1,4-benzoquinone ^(b)
J	1346	152 (M^+ , 68), 137 (14), 124 (75), 123 (19), 122 (66), 109 (22), 96 (15), 94 (12), 81 (6), 69 (100), 66 (27), 53 (26)	2-methoxy-6-methyl-1,4-benzoquinone ^(b)
K	1411	154 (M^+ , 100), 139 (6), 128 (6), 126 (58), 111 (20), 98 (12), 97 (16), 85 (8), 82 (39), 72 (9), 57 (22), 54 (27)	2-hydroxy-3-methoxy-1,4-benzoquinone ^(c)
L	1419	182 (M^+ , 97), 167 (42), 153 (16), 139 (24), 137 (100), 136 (21), 121 (7), 111 (19), 108 (7), 96 (7), 83 (24), 69 (13), 68 (17), 67 (7)	2,3-dimethoxy-5-methyl-1,4-benzoquinone
M	1455	184 (M^+ , 95), 169 (83), 152 (45), 126 (24), 121 (100), 93 (23), 69 (13), 65 (15)	mixed spectrum: 2,3-dimethoxy-5-methylhydroquinone + methyl-p- <i>paraben</i> ^(d)
N	1455	152 (M^+ , 50), 122 (6), 121 (100), 93 (16), 92 (3), 65 (7)	methyl-p- <i>paraben</i> (<i>p</i> -hydroxybenzoic acid methyl ester)
O	1499	184 (M^+ , 52), 169 (100), 140 (7), 139 (12), 127 (14), 123 (25), 113 (23), 87 (18), 85 (42), 72 (11), 69 (7), 68 (6), 54 (6)	dimethoxy-hydroxy-benzoquinone isomer ^(c)
P	1518	168 (M^+ , 100), 153 (21), 140 (61), 125 (22), 112 (7), 97 (16), 96 (10), 85 (20), 72 (12), 68 (33)	2-hydroxy-3-methoxy-5-methyl-1,4-benzoquinone ^(c)
Q	1524	170 (M^+ , 100), 169 (78), 142 (12), 141 (36), 139 (10), 115 (21), 89 (3)	2-phenylphenol
R	1532	214 (M^+ , 100), 169 (11), 168 (35), 167 (12), 155 (19), 138 (51), 137 (21), 136 (17), 127 (75), 114 (6), 99 (26), 68 (17), 59 (17)	unidentified
S	1598	200 (M^+ , 100), 185 (10), 151 (42), 139 (7), 123 (12), 85 (2), 69 (3)	unidentified
T	1606	198 (M^+ , 100), 183 (86), 170 (2), 165 (28), 155 (15), 153 (9), 152 (4), 137 (32), 127 (20), 123 (4), 109 (8), 99 (26), 96 (6), 87 (19), 83 (5), 72 (4), 68 (9)	dimethoxy-hydroxy-methyl-benzoquinone isomer 1 ^(e)
U	1680	198 (M^+ , 100), 183 (18), 180 (4), 170 (8), 165 (6), 155 (26), 152 (7), 140 (3), 137 (4), 127 (12), 125 (3), 112 (5), 109 (8), 99 (18), 85 (12), 83 (12), 72 (4)	dimethoxy-hydroxy-methyl-benzoquinone isomer 2 ^(e)
V	1691	198 (M^+ , 100), 183 (55), 180 (11), 170 (20), 169 (18), 165 (7), 155 (11), 151 (5), 140 (2), 137 (4), 127 (14), 125 (37), 123 (1), 112 (7), 109 (2), 99 (33), 85 (26), 83 (24), 82 (1), 72 (1), 67 (4)	dimethoxy-hydroxy-methyl-benzoquinone isomer 3 ^(e)
W	1932	230 ($[M + 2]^+$, 59), 228 (M^+ , 100), 213 (19), 211 (11), 199 (15), 185 (24), 159 (13), 129 (19), 119 (27), 110 (17), 91 (35), 69 (30), 65 (31), 53 (26)	C ₁₄ H ₁₃ O ₅ ^(e)

^a Radulović et al. (2010). ^b Isomers assigned according to Wu et al. (2007). ^c Tentatively identified on the basis of mass spectral fragmentation and high resolution mass spectrometry. ^d Peak M was specific for *Brachyulius lusitanus*, containing 2 compounds, namely 2,3-dimethoxy-5-methylhydroquinone and methyl-p-*paraben* in a ratio of about 1:1. RIs measured for authentic 2,3-dimethoxy-5-methylhydroquinone and methyl-p-*paraben*, respectively, proved to be indistinguishable (for both compounds RI 1455), and co-injection of both compounds (in 1:1 ratio) led to a single, sharp peak, showing a mass spectrum as observed for peak M. On the other hand, methyl-p-*paraben* (without 2,3-dimethoxy-5-methylhydroquinone) was found in the extracts of *Typhlotritus* n. sp. (peak N). ^e Molecular formula, based on high resolution mass measurement

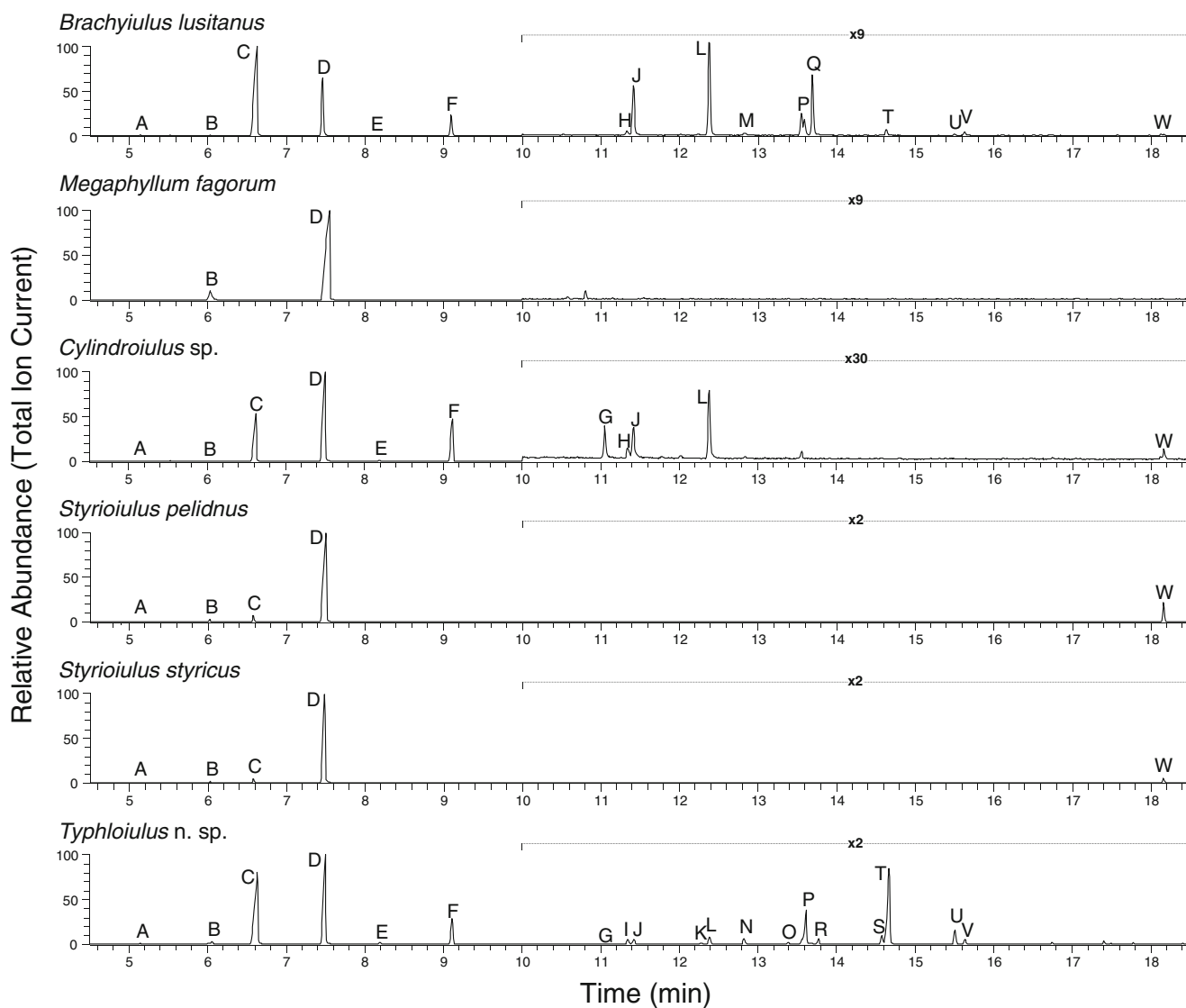


Fig. 1 Gas chromatographic profiles of the defensive secretions of phenol-producing julid species. Phenolic compounds are: peak B (phenol), peak D (*p*-cresol). For a complete list of compounds see

Table 2. Note that the chromatographic response between 10.00 and 18.50 min retention time is amplified for a better demonstration of minor peaks

Compounds N and Q were identified as methyl-paraben and 2-phenylphenol, respectively. Compound W was partly identified: it exhibited a molecular ion at m/z 228 (base peak), together with an intense $M + 2$ -ion, indicating the reduction of a quinone in the hot injector. The compound was first suspected to represent a 2,3,5,6-tetramethoxy-1,4-benzoquinone (TM-BQ, $C_{10}H_{12}O_6$), but a comparison to authentic TM-BQ showed distinctly different retention times and RIs, respectively (measured $RI_{TM-BQ} = 1713$; measured $RI_{compound\ W} = 1932$). High resolution mass spectrometry via HPLC-MS led to a probable elemental composition of $C_{14}H_{12}O_3$ (measured monoisotopic mass: 228.0786; $R = 140.000$; theoretical monoisotopic mass: 228.0786). This particular molecular formula indicates a highly condensed component (rings plus double bonds = 9), and thus, most likely 2 ring systems. The detailed structure of this compound remained unknown.

Compounds K, O, P, T, U, and V were not fully identified. These compounds shared the mass spectrometric characteristics of hydroxy-methoxy-1,4-benzoquinones (Budzikiewicz et al. 1967: p. 530). Possible structures, supported by high resolution mass spectrometry, are proposed in Table 2. The detailed chemical identification of these compounds will be presented in a separate paper. Compounds R and S remained unidentified.

Secretion Profiles The 6 phenol-producing species exhibited highly consistent secretion profiles in terms of profile quality (= compound composition) as well as relative abundance of components. The chromatographic profiles are given in Table 3. Only the two species of *Styrioiulus* showed indistinguishable profiles, and a comparison of individual profiles based on non-metric multidimensional scaling (NMDS)

Table 3 Gas chromatographic profiles* of defensive secretions of phenol-producing Julidae

Peak no.	compound	<i>Brachyiulus lusitanus</i>	<i>Megaphyllum fagorum</i>	<i>Cylindroiulus sp.</i>	<i>Styrioiulus pelidnus</i>	<i>Styrioiulus styricus</i>	<i>Typhloiulus n.sp.</i>
A	1,4-benzoquinone	trace	-	0.2	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
B	phenol	trace	7.8	0.1	1.3 ± 0.5	2.1 ± 0.8	0.7 ± 0.1
C	2-methyl-1,4-benzoquinone	54.3 ± 6.2	-	25.2	3.5 ± 1.1	3.8 ± 1.0	31.8 ± 4.2
D	p-cresol	21.4 ± 9.7	92.2	50.6	93.1 ± 2.0	93.0 ± 1.6	31.8 ± 6.3
E	2-hydroxy-3-methyl-1,4-benzoquinone	0.5 ± 0.5	-	0.5	-	-	0.4 ± 0.1
F	2-methoxy-3-methyl-1,4-benzoquinone	16.2 ± 10.7	-	20.9	-	-	8.1 ± 1.8
G	2,3-dimethoxy-1,4-benzoquinone	-	-	0.5	-	-	trace
H	2-methylhydroquinone	0.1 ± 0.1	-	0.2	-	-	-
I	2-methoxy-5-methyl-1,4-benzoquinone	-	-	-	-	-	0.7 ± 0.2
J	2-methoxy-6-methyl-1,4-benzoquinone	1.9 ± 0.8	-	0.5	-	-	0.6 ± 0.2
K	2-hydroxy-3-methoxy-1,4-benzoquinone	-	-	-	-	-	0.2 ± 0.1
L	2,3-dimethoxy-5-methyl-1,4-benzoquinone	3.9 ± 2	-	1.1	-	-	1.3 ± 0.2
M	2,3-dimethoxy-5-methylhydroquinone + methyl-paraben	0.3 ± 0.1	-	-	-	-	-
N	methyl-paraben	-	-	-	-	-	0.8 ± 0.2
O	dimethoxy-hydroxy-benzoquinone isomer	-	-	-	-	-	0.2 ± 0.1
P	3-hydroxy-5-methoxy-5-methyl-1,4-benzoquinone	0.3 ± 0.3	-	-	-	-	5.4 ± 0.6
Q	2-phenylphenol	1.1 ± 4.1	-	-	-	-	-
R	unidentified	-	-	-	-	-	0.6 ± 0.2
S	unidentified	-	-	-	-	-	1.2 ± 0.1
T	dimethoxy-hydroxy-methyl-benzoquinone isomer 1	0.1 ± 0.2	-	-	-	-	13.6 ± 1.6
U	dimethoxy-hydroxy-methyl-benzoquinone isomer 2	trace	-	-	-	-	1.8 ± 0.2
V	dimethoxy-hydroxy-methyl-benzoquinone isomer 3	trace	-	-	-	-	0.7 ± 0.1
W	C ₁₄ H ₁₃ O ₃	trace	-	0.1	2.0 ± 1.7	1.1 ± 0.7	-

*Compounds are given as % peak area of whole secretion as described in material & methods; thus, each column represents the chemical secretion profile of a species. Specific profiles (including means and standard variations for each compound) are based on the examination of 23 individuals for *Brachyiulus lusitanus*, a pooled extract (3 individuals) of *Megaphyllum fagorum*, 1 individual of *Cylindroiulus sp.*, 24 individuals of *Styrioiulus pelidnus*, 23 individuals of *Styrioiulus styricus*, and 10 individuals of *Typhloiulus n. sp.* Phenolic compounds in bold

resulted in a scatter plot, showing one single cluster (Fig. 2a). All other species were readily distinguishable by their characteristic secretion chemistry. For *B. lusitanus*, individuals from the same location exhibited slightly different but statistically distinguishable secretion patterns (Fig. 2b) in a repeated investigation after a time interval of one year. Statistically significant differences between female and male secretion pattern were not observed for any of the species.

Discussion

Phenols in Juliformian Millipedes? Although *p*-cresol and phenol have been reported sporadically as minor compounds in the quinone-rich secretions of julids, such as *Unciger transsilvanicus* (Uncigerini) (Sekulić et al. 2014), and are indicated for several species of *Serboiulus* (Leptoiulini), we here provide the first examples for clearly phenol-based chemical secretions (= phenolic content of secretion >20 %) in the

family Julidae. In three species analyzed, phenolic content was higher than 90 %: in *S. pelidnus* and *S. styricus* about 95 %, and in *M. fagorum* the secretions were exclusively phenolic. Most interestingly, these phenol-producing species are not closely related, and the distribution of phenols does not follow an obvious taxonomic pattern. Species with phenolic secretions appear to be unexpectedly present in genera or tribes whose “normal” representatives rely on the common benzoquinonic chemistry. We found such “aberrant”, phenol-producing species in 5 genera, belonging to 3 different tribes of julids - Brachyiulini, Cylindroiulini, and Leptoiulini. So far, each of these tribes was known for uniform benzoquinonic chemistry. Taking the example of Cylindroiulini, hitherto analyzed species of *Cylindroiulus* (Huth 2000), *Enantiulus* (Huth 2000), and *Allajulus* (Bodner and Rasputnig 2012) showed non-phenolic, benzoquinone-dominated secretions. Comparably, preliminary data indicate that the mono-specific cylindroiuline genus *Kryphioiulus* exclusively discharges benzoquinones as well. Thus, phenols appear to have arisen at least twice in cylindroiulines,

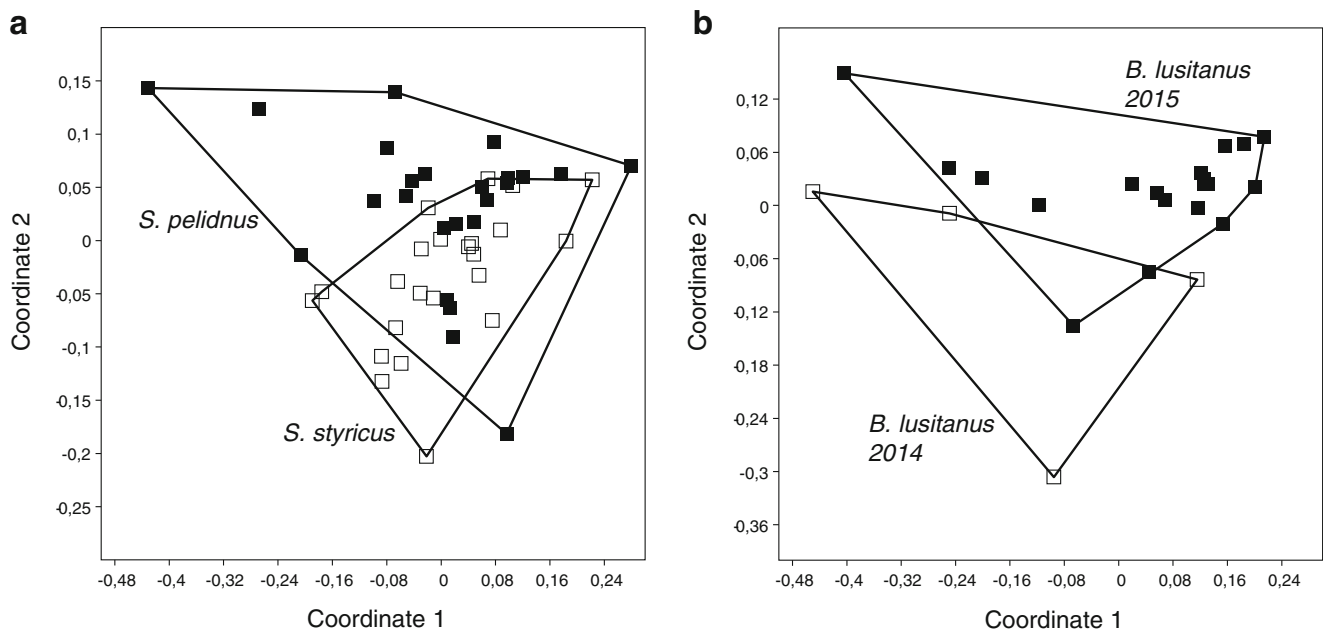


Fig. 2 Statistical discrimination of chemical profiles of (a) individuals of *Styrioiulus pelidnus* and *S. styricus*, and (b) individuals of *Brachyiulus lusitanus* from 2 collections. Plots based on NMDS using the Bray Curtis coefficient

once in *Styrioiulus* and a second time in a lineage of *Cylindroiulus*. In this respect, it is important to recall the paper of Kluge and Eisner (1971), who reported on a phenol-rich secretion in the parajulid *Oriulus venustus*. Until now, this was the only report on a phenol-predominated secretion in the Juliformia. Assessing this report in light of new data, there is evidence that phenolic secretions are not exceptional in the Julida. Taking current data on julidan secretions as a basis (including literature data), we can currently list 7 species with phenol-dominated secretions out of 57 chemically investigated species. These belong to families Julidae, Blaniulidae, and Parajulidae (Table 1). If we include those species with phenols as minor compounds (3 additional species, Table 1), then phenol-producers comprise about 18 % of the Julida so far investigated. These data indicate that phenolic secretions among the Julida are no rarity and that they are to be expected in a number of additional taxa.

Phenol Biosynthesis and Evidence for a Shared Phenol Origin

Both benzoquinones and phenols are widespread compounds in the defensive secretions of arthropods. High selective pressure may have led to independent evolution of such compounds in groups such as Coleoptera, Blattaria, Dermaptera, Opiliones, and Diplopoda (Blum 1981, 1996). Additionally, within distinct taxa, it frequently is not clear whether phenolics and benzoquinones may have arisen independently in different sub-taxa, or whether they share a common ancestry (e.g., Caetano and Machado 2013; Raspotnig et al. 2015). In order to address such questions, information on the biosynthetic pathways leading to the compounds is important (Blum 1981). There are only a few studies dealing with

the biosynthesis of phenols and benzoquinones in arthropods. In harvestmen, for instance, the biosynthesis of phenols and benzoquinones follows a common multi-step pathway, in the course of which the condensation of acetate and propionate units leads to phenols that subsequently give rise to benzoquinones via para-oxidation (Raspotnig et al. 2015; Rocha et al. 2013). Insects may produce benzoquinones differentially, but nevertheless retain the step of phenol oxidation: for example, the tenebrionid beetle *Eleodes longicollis* produces 1,4-benzoquinone by oxidation of dihydroxybenzene (1,4-hydroquinone), which in turn arises from arbutin, a hydroquinone-glucopyranosid (Happ 1968). Regarding millipedes, it has been assumed that benzoquinone synthesis relies on the availability of aromatic amino acids (Blum 1981). The polydesmidan *Oxidus gracilis*, for instance, produces HCN, benzaldehyde, but also phenol, guaiacol, and arbutin from tyrosine by tyrosine phenol lyase (Duffey and Blum 1977). Based on the occurrence of phenol tyrosine lyase in juliformian millipedes, Duffey and Blum (1977) suggested a similar biosynthetic pathway to juliformian benzoquinones, basically leading to phenolics, then to hydroquinones via arbutin, then to benzoquinones via paraoxidation.

Evidence for Ancestral Chemical Equipment? Even though the putative sister group of the Juliformia is still in discussion (Enghoff 1984; Sierwald et al. 2003) there is evidence that close juliformian outgroups may be represented by Nematophora (comprising Callipodida, Stemmiulida, and Chordeumatida) and Polydesmida (Blanke and Wesener 2014; Miyazawa et al. 2014; Sierwald and Bond 2007).

Interestingly, in these outgroups, the production of *p*-cresol is widespread or even characteristic (Shear 2015). Phenol biosynthesis requires a complex machinery of different enzymes, making the multiple independent development of the same compounds in closely related taxa less likely. Following this argument, phenolics may be considered the common, ancestral chemical components of juliformian, nematophoran, and polydesmidan defensive secretions. Particularly callipodidan secretions (generally phenol as minor, and *p*-cesol as major constituent) are reminiscent of findings for the Julidae (Ćurčić et al. 2009; Eisner et al. 1963; Makarov et al. 2011; Shear 2015; Shear et al. 2007, 2010).

With respect to Juliformia and to Julida in particular, we thus hypothesize that benzoquinones arose from the ancestral state of phenolics, and that the oxidation-step to benzoquinones evolved later, possibly in early juliformians. This event led to the replacement of phenolics by quinones in most juliformian taxa. Benzoquinones thus may be younger than phenolics, and possibly arose in early juliformians before the split into orders Spirostreptida, Spirobolida, and Julida. This situation basically supports a scenario for diplopod chemosystematics as recently discussed by Shear (2015).

We thus consider the most likely explanation for the patchy, non-taxonomic distribution of phenol-based secretions across the Julida (and presumably in the remaining Juliformia) to be the result of the loss of the para-oxidation step from phenols to benzoquinones, i.e., the last step in the proposed common multi-step pathway to benzoquinone formation (Rasputnig et al. 2015). This loss may occur with the inactivation of a single enzyme, and is thus a parsimonious explanation compared to the possibility of multiple independent evolutions of multi-step pathways to phenolics in different juliformian taxa.

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