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1 **Metabolomic variability of four macroalgal species of the genus *Lobophora* using diverse**
2 **approaches**

3

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13 **Abstract**

14

15 Among comparative metabolomic studies used in marine sciences, only few of them are dedicated to macroalgae
16 despite their ecological importance in marine ecosystems. Therefore, experimental data are needed to assess the
17 scopes and limitations of different metabolomic techniques applied to macroalgal models. Species of the genus
18 *Lobophora* belong to marine brown algae (Family: Dictyotaceae) and are widely distributed, especially in
19 tropical coral reefs. The species richness of this genus has only been unveiled recently and it includes species of
20 diverse morphologies and habitats, with some species interacting with corals. This study aims to assess the
21 potential of different metabolomic fingerprinting approaches in the discrimination of four well known
22 *Lobophora* species (*L. rosacea*, *L. sonderii*, *L. obscura* and *L. monticola*). These species present distinct
23 morphologies and are found in various habitats in the New Caledonian lagoon (South-Western Pacific). We
24 compared and combined different untargeted metabolomic techniques: liquid chromatography-mass
25 spectrometry (LC-MS), nuclear magnetic resonance (¹H-NMR) and gas chromatography (GC-MS).
26 Metabolomic separations were observed between each *Lobophora* species, with significant differences according
27 to the techniques used. LC-MS was the best approach for metabotype distinction but a combination of
28 approaches was also useful and allowed identification of chemomarkers for some species. These comparisons
29 provide important data on the use of metabolomic approaches in the *Lobophora* genus and will pave the way for
30 further studies on the sources of metabolomic variations for this ecologically important macroalgae.

31

32 **Keywords:** *Lobophora* – Dictyotaceae – macroalgae – metabolomic – fingerprinting – comparative approach –
33 New Caledonia

34

35 1.Introduction

36 Specialized metabolites are often considered as low molecular weight molecules, end products of cellular
37 regulatory processes, and final responses of biological systems to genetic and/or environmental changes (Fiehn,
38 2002). They can be regarded as products of natural selection during evolution. These secondary metabolites play
39 also an important role in shaping algal chemical diversity (Wink, 2003). The set of metabolites present in the
40 organisms can be highly complex and their biosynthesis can also be related to the associated microbiota
41 (Roessner and Bowne, 2009). Traditionally, the chemical composition of an organism is explored through
42 natural product chemistry which includes long and tedious steps of isolation and structure elucidation of
43 metabolites (Robinette et al., 2011). This approach is time consuming and incomplete as it focuses mostly on the
44 major compounds produced. Recent advances in more global approaches called metabolomics allow the analysis
45 of a wider part of the metabolome by the simultaneous detection of hundreds to thousands of the metabolites of a
46 small sample in a short period of time. In environmental sciences, metabolomics has therefore appeared as a
47 quick and useful approach to examine the metabolite diversity of species and study their variations with time,
48 geography, biotic interactions or other environmental factors (Bundy et al., 2009). Compared to the plant
49 kingdom, relatively few environmental metabolomics studies have been reported on marine organisms.
50 Taxonomy-based metabolomics has been applied for marine organisms like sponges (Ivanišević et al., 2011a;
51 Pérez et al., 2011), zoanthids (Costa-Lotufu et al., 2018; Jaramillo et al., 2018) and microalgae (Mooney et al.,
52 2007). Variability in the metabolomic profiles were explored in time and space for some sponges (Rohde et al.,
53 2012), ascidians (López-Legentil et al., 2006), zoanthids (Cachet et al., 2015) and corals (Slattery et al., 2001)
54 but also in response to environmental factors like temperature or salinity (Abdo et al., 2007; Bussell et al., 2008).

55 Among the chemical studies dedicated to macroalgae, only a few used metabolites as a taxonomic tool targeting
56 specific compounds or classes like phenolics (Connan et al., 2004) or diterpenes (Campos De Paula et al., 2007).
57 While these studies traditionally focus on potentially active compounds with pharmaceutical interests, a more
58 global approach using metabolomics can represent a useful tool to explore the metabolome of macroalgae and its
59 fluctuations. For example, metabolomics was applied on the red alga *Asparagopsis taxiformis* to study the
60 spatio-temporal variation of its metabolome (Greff et al., 2017). Another study on the red alga *Portieria*
61 *hornemannii* explored different sources for the variation of non-polar metabolites between cryptic species and
62 life stages (Payo et al., 2011). Metabolomics also appeared as a complementary tool to understand defense or
63 tolerance mechanisms of macroalgae in an ecological context (Rempt et al., 2012; Ritter et al., 2014). Marine
64 brown macroalgae from the genus *Lobophora* (Family Dictyotaceae) have already been studied chemically.
65 Gerwick & Fenical (1982) first described 1-(2,4,6-trihydroxyphenyl)hexadecan-1-one in *L. papenfussii*. Three
66 sulfoquinovosyldiacylglycerols (SQDGs) and later lobophorolide were identified from *L. variegata* (Cantillo-
67 Ciau et al., 2010; Kubanek et al., 2003). Recently, seven nonadecaketides named lobophorols, lobophopyranones
68 and lobophorones (Gutiérrez-Cepeda et al., 2015) were found in the Atlantic *L. variegata* while the
69 polyunsaturated lobophorenols A, B and C were described in the tropical *L. rosacea* (Vieira et al., 2016).
70 Abundant in tropical coral reef habitats, some *Lobophora* species are closely associated with corals and therefore
71 strongly involved in coral-algal interactions (Rasher & Hay, 2010), leading in some cases to negative impacts on
72 corals. The high specific diversity of *Lobophora* genus has recently been unveiled (Vieira et al., 2014, 2017),
73 with species exhibiting various morphologies and habitats, questioning the link between chemical diversity and

74 species diversity. Due to this chemical diversity, species of this genus are therefore good candidates to undergo
75 metabolomics-based study to explore the metabolic variability among the different species.

76 We first decided to assess the potential of different approaches in metabolomic fingerprinting to separate four
77 well-known *Lobophora* species (*L. rosacea*, *L. sonderii*, *L. obscura* and *L. monticola*), with distinct morphology
78 and present in diverse habitats of the New Caledonian lagoon (South-Western Pacific). The systematics of these
79 species being well described, we aimed at providing important insights on the relevance of these approaches to
80 first discriminate species. The results of these preliminary data will then pave the way for deeper metabolomic
81 studies on the presence of cryptic species, the influence of environmental parameters or biotic factors like the
82 reproductive cycles. In terms of reproduction, little is known about *Lobophora* in New Caledonia and this genus
83 is supposed to be reproductive all year round (Vieira, pers. com.). Our knowledge on the main specialized
84 metabolites found in *L. rosacea* was a prerequisite to guide our study as they are presumed to have a taxonomic
85 relevance. We used untargeted metabolomic approaches using three different techniques: UHPLC-MS-QToF,
86 ¹H-NMR and GC-MS followed by unsupervised and supervised analyses to highlight chemical differences
87 among species.

88

89 **2.Results**

90 **2.1.¹H-NMR**

91 The matrix obtained after data analyses was composed of 7,998 buckets. A value of 33.5% of variance was
92 explained by the two first components of the PCA (Fig. 1a) and mainly due to *L. rosacea* characterized by a
93 different metabolomic fingerprint than the other three species (PLS-DA, CER = 0.328, p = 0.001, post hoc p <
94 0.05, Fig. 1b, Table S1). In the central cluster of the PCA that grouped the three other species, only *L. monticola*
95 and *L. obscura* present significant different metabolomic fingerprints (p = 0.018), whereas *L. sonderii* is not
96 chemically different from the two others (Table S1, p > 0.05).

97 The overlay of ¹H NMR spectra (Fig. 2), indicated that the major signals are shared by all *Lobophora* species:
98 intense signals at δ_H 1-2 ppm due to the methylenes of long chain fatty acids, and signals at δ_H 2.8 and 5.3 ppm
99 attributed to carbon-carbon unsaturations. More variable regions containing characteristic signals of the
100 polyunsaturated lobophorenols A, B and C were observed between δ_H 3.2-4.5 (chlorinated and hydroxylated
101 methines), 4.8-5.2 and 5.0-5.8 ppm (terminal olefinic protons, Fig. 2 and S1). Due to the high number of
102 generated bins, Kruskal-Wallis loading plot (Fig. S2) was used to identify chemical markers, which separate
103 metabolic diversity of *Lobophora* species (with p < 0.05, Table S2). The regions corresponding to the signals of
104 lobophorenols are the main markers of differences between species and are mostly present in *L. rosacea*.

105

106 **2.2.UHPLC-QToF**

107

108 After LC-MS data analyses and filtering, 600 metabolic features were finally considered. The variance on the
109 two first components of the PCA was explained by 38.7% (Fig.1c), a value slightly higher than for NMR
110 analysis. The LC-MS approach permitted a better separation of each species' metabolome than NMR (CER =

111 0.115, $p = 0.001$, $p < 0.05$ for each tested pair, Fig. 1d, Table S1). The difference between chemical groups was
112 mainly quantitative as shown in the Venn diagram (Fig. S4).

113 Among chemomarkers, two compounds, lobophorenol B (m/z 334.272 $[M + NH_4]^+$, $C_{21}H_{32}O_2$) and lobophorenol
114 C (m/z 336.287 $[M + NH_4]^+$, $C_{21}H_{34}O_2$), previously isolated in *L. rosacea* (Vieira et al., 2016) were mainly
115 detected in this species (Fig. 3, Table S3). They were also detected in *L. monticola*, but with high variability and
116 not in *L. sonderii* and *L. obscura*. The other chemomarkers of each species were tentatively annotated based on
117 the construction of a molecular network but no other match was found in the current database, with the majority
118 of them appearing as minor intensity ions.

119 To combine data obtained by LC-MS with those by NMR, a multiple factor analysis (MFA) was performed (Fig.
120 4). The four *Lobophora* species were well separated despite a lower variance of 21.7% on the two first
121 dimensions.

122

123 2.3. GC-MS

124 The richness in non-polar specialized metabolites in *Lobophora* led us to analyze the CH_2Cl_2 fractions by GC-
125 MS while attempts to use NMR for those fractions were unfruitful due to intense lipidic peaks. The explained
126 variance on axis 1-2 of the PCA was 35.7%, a value similar to the values obtained with the two other techniques
127 (Fig. 1e). All algal metabotypes were differentiated with this technique (CER = 0.304, $p = 0.001$, Fig. 1f) except
128 for *L. monticola* vs *L. rosacea* ($p = 0.431$, Table S1). Among the chemomarkers contributing to the
129 discrimination of the species metabotypes, we identified a small carboxylic acid: 2-pentenoic acid (M5), an
130 amide: maleimide, 2-methyl-3-vinyl (M18), and two esters: methyl stearate (M38) and hexanoic acid, 2-ethyl-,
131 hexadecyl ester (M48) (Table 1). M5 and M48 are specific to *L. obscura* while this species contained lower
132 amount of M38 compared to the three other species. *Lobophora sonderii* and *L. rosacea* exhibit higher levels of
133 M18 (Fig. S5).

134 Moreover, some compounds known as plastic pollutants were found in all species and contribute to differences
135 in their chemical profiles (Phenol, 2,4-di-*tert*-butyl; tributyl acetyl citrate; *o*-xylene; naphthalene, 2,6-dimethyl-;
136 *p*-cresol, 2,6-di-*tert*-butyl-; *N*-methyl-*N*-benzyltetradecanamine).

137

138 3. Discussion

139

140 Even though LC-MS is a method largely employed in metabolomics studies due to its high sensitivity, it is not
141 suitable for all metabolites and more appropriate for polar, weakly polar and neutral compounds (Wang et al.,
142 2015). Moreover, it relies on ionization process, limiting the study of poorly-ionizable compounds. GC-MS is
143 then more suitable when non-polar metabolites are found as the main major specialized metabolites. On the
144 contrary and even if much less sensitive, NMR is more universal and does not rely on ionization processes nor
145 the separation of analytes by HPLC, and solubilization of the metabolites is the only limitation. NMR spectra are
146 able to provide a better snapshot of what are the major metabolites and their relative concentrations in the
147 studied specimens. This information is highly relevant for major metabolites that may correspond to specialized

148 metabolites providing useful information concerning species discrimination. NMR would therefore be less
149 affected by environmental changes often linked to minor metabolites (Ivanišević et al., 2011b).

150 The four species of *Lobophora* are well described morphologically and their phylogeny resolved in 2014 using
151 mitochondrial gene Cox3 (Vieira et al., 2014). *Lobophora monticola*, *L. obscura*, *L. rosacea* and *L. sonderii* are
152 distantly-related species. They are distributed evenly across the *Lobophora* evolutionary tree, with *L. rosacea* the
153 most basal species of the four (Fig. S6)(Vieira et al., 2014, 2017). In the current study, the algal species have
154 been separated based on their metabolomic fingerprints, and depending on the used technique. The LC-MS
155 approach was the most effective technique for the separation of the four species of this genus (PLS-DA, CER =
156 0.115), but NMR and GC-MS (CER_{NMR} = 0.328, CER_{GC/MS} = 0.304) also provided interesting complementary
157 results. While less sensitive than LC-MS or GC-MS, NMR provides the highest reproducibility among
158 metabolomic measuring platforms (Frag et al., 2012a).

159 In our study, NMR analyses performed on the same methanolic fractions as LC-MS allowed a clear distinction
160 of *L. rosacea* from the other three species, in agreement with phylogenetic data where this species appears as the
161 most basal. Like LC-MS, NMR highlighted lobophorenols as discriminating metabolites in *L. rosacea*, with
162 most of the signals responsible for the chemical divergence corresponding to characteristic signals of these
163 molecules. Lobophorenol A was not detected by LC-MS while it was observed as marker in NMR because of
164 some characteristic signals at δ_H 3.70 (H-4) and 6.02 ppm (H-2; see Fig. S1). The absence of detection of
165 lobophorenol A by LC-MS might stem from a high reactivity of the chlorinated derivative during ionization.
166 These lobophorenols have been shown to present allelopathic effects against the coral *Acropora muricata* (Vieira
167 et al., 2016). Molecular networking based on MS² spectra analyses did not allow identification of additional
168 chemomarkers. Annotation of compounds from marine organisms, especially macroalgae, is still challenging
169 with the lack of specific databases. Altogether, LC-MS and NMR provide complementary approaches to analyze
170 the metabolome and their combination is highly relevant to discriminate *Lobophora* species, as supported by the
171 MFA.

172

173 NMR analyses also showed the rich composition in lipidic derivatives of *Lobophora* species, which may be
174 problematic because intense long-fatty-chain signals may mask other signals. However, this issue could be
175 resolved using GC-MS, allowing the study of the non-polar part of the metabolome. Compared to the poor
176 specialized LC-MS database, the available GC-MS databases enabled the annotation of some chemomarkers.
177 However, the discrimination between species metabolotypes was partly explained by compounds presumably
178 identified as pollutants from plastic origin and present in the coastal sites, which were likely differently
179 accumulated in the algae or at their surface as seen in *Sargassum* spp. and *Fucus vesiculosus* (Chan et al., 2004;
180 Gutow et al., 2016). This result potentially highlights more different adhesion capacities of microplastics
181 between species rather than metabolic differences. Other markers evidenced by the NMR method include: 2-
182 pentenoic, which is a small unsaturated fatty acid previously found in plants (Wu and Chen, 1992). Pentenoic
183 acid was found up-regulated under salinity stress in the halophyte *Aeluropus lagopoides* (Paidi et al., 2017).
184 Maleimide, 2-methyl-3-vinyl was also detected in the algal samples. This metabolite may be a transformation
185 product of chlorophylls and bacteriochlorophylls (Naeher et al., 2013). We hypothesize that these compounds
186 originate in part from micro-organisms associated to *Lobophora* species. Indeed, epiphytes often colonized the

187 algal surface (Egan et al., 2013). Then, by extracting the algal metabolome, we may also extract compounds
188 from bacteria or epiphytes and metabotypes observed in our study may arise, at least in part, from the algal-
189 associated organisms. Even if we carefully removed epiphytes from their surface, microorganisms are still
190 present and may contribute to the global metabolome of the specimens. For example, methyl stearate has been
191 found in bacteria and plants but also microalgae, ascidians and macroalgae (De Rosa et al., 2001; Sharmin et al.,
192 2016; Takeara et al., 2008; Terekhova et al., 2010) and is assumed to have antibacterial and cytotoxic activities
193 (Elshafie et al., 2017; Takeara et al., 2008). The influence of species-specific microbial communities, which can
194 produce minor compounds, may also explained the better result obtain by LC-MS in the discrimination of
195 *Lobophora* species.

196 Even if less important than interspecific differences, intraspecific variability can be explained by their
197 development stage, life history traits and evolution and may also result from their environment. *Lobophora* life
198 cycle is not documented in New Caledonia lagoon and reproductive state has been seen all year round (Vieira,
199 personal observation). Gametophytes and sporophytes are not easily dissociable but may potentially present
200 different chemical profiles as seen in other macroalgae, like the red algae *Portieria hornemannii* in the
201 Philippines (Payo et al., 2011). The close association of *L. rosacea* and *L. monticola* with corals could lead to
202 chemical adaptation or specification in the algal chemistry, notably against coral associated microbiome. On the
203 other hand, growing in algal beds, *L. sonderii* is more exposed to herbivores and its chemistry probably evolved
204 differently, notably to repulse predators. *Lobophora obscura* is also exposed to other organisms but its
205 encrusting form with thick and coarse thalli, may deter predators. Less effort in metabolites production may be
206 balanced by its protective morphology. Because very few chemomarkers were identified, these hypotheses
207 should be further investigated and tested. Moreover, regarding the good discrimination observed with LC-MS in
208 this work, this method may be useful to separate cryptic species of *Lobophora*, as successfully applied for
209 *Portieria dioli* (Payo et al., 2011).

210 The choice of the technique to accurately distinguish species is pivotal and only few studies comparing these
211 techniques have been published to date, most of them being applied to plants. For example, untargeted LC-MS
212 was the most effective to discriminate several green tea (Kellogg et al., 2017). Other authors used multiple
213 approaches to study the metabolomic fingerprint in zoanthids (Costa-Lotufu et al., 2018) or in the plant kingdom
214 (Agnolet et al., 2010; Farag et al., 2012b). Multiple metabolomics approaches are rare on macroalgae. Notably,
215 LC-MS and HR-MAS NMR were used to evaluate the relevance for taxonomical purpose in five species of the
216 genus *Cystoseira* (Jégou et al., 2010). Due to the high diversity in metabolites, with diverse physico-chemical
217 properties and different concentration ranges, the global analysis in metabolites is challenging. Using multiple
218 metabolomics approaches allow a broader analysis. It is a good tool to appreciate the chemical diversity among
219 species and can bring complementary information to the phylogenetic data, the unavoidable base for
220 classification. These approaches enabled a better exploration of the chemical speciation or evolution among
221 genus or even at a broader scale, as realized by Belghit et al.(2017) on 21 species belonging to red, green and
222 brown algae. With the increase of shared metabolomics platforms, metabolomic fingerprinting might be applied
223 to other macroalgae and marine organisms, and when coupled with genomics or transcriptomics, it will greatly
224 improve our understanding of adaptive mechanisms involved in multi-stressors environments. This coupling has
225 been recently applied to macroalgae, like in the model *Ectocarpus siliculosus* where transcriptomic and genomic
226 data available allowed to better understand the metabolic changes during saline and oxidative stress (Dittami et

227 al., 2011) or under different CO₂ and O₂ concentrations (Gravot et al., 2010). While *Lobophora* genus is not a
228 typical model organism, the decreased cost and increased sequencing capabilities of Next Generation
229 Sequencing make it possible to examine species beyond traditional models (Konotchick et al., 2013; Unamba et
230 al., 2015). In particular, it is an example of common brown alga widely distributed in tropical waters and
231 producing major non-polar metabolites and therefore can represent a model for other metabolomic studies
232 applied to brown algae. A coupling between metabolomics and meta-genomics could also help to understand the
233 diversity of associated bacteria and better assess their contribution to the algal metabolome. Because associated
234 microorganisms are commonly species-specific, this could partly explain the better results obtained with LC-MS.
235 While a multiple metabolomic approach is promising for several applications in macroalgae, data interpretation
236 remain the biggest challenge to date and more metabolomics studies on macroalgae are needed.

237

238 **4. Conclusion**

239 Metabolic fingerprinting with LC-MS was the most appropriate technique in the discrimination of different
240 *Lobophora* species, but the coupling with NMR is also useful as the main metabolites can be observed with these
241 methods and identified as chemomarkers. Indeed, lobophorenols, previously identified specialized metabolites in
242 *L. rosacea*, were detected as chemomarkers with both LC-MS and NMR while they were not detected by GC-
243 MS, which appeared a less useful technique for analyzing the *Lobophora* genus. This study demonstrates that an
244 untargeted metabolomic approach via LC-MS/NMR will be helpful for further ecological studies in *Lobophora*.
245 Notably, this technique is appropriate to explore the sources of metabolomic variations in this genus at the
246 temporal and spatial scales, influenced by environmental factors, and also in response to different biotic
247 interactions.

248

249 **5. Experimental**

250 **5.1. Sampling**

251 *Lobophora rosacea*, *L. sonderii* and *L. obscura* were collected by SCUBA during summer 2016 at Ricaudy
252 (22°18.956'S; 166°27.405'E, Nouméa, New Caledonia). *Lobophora monticola* was collected during summer
253 2016 at Sainte-Marie (22°18.269'S; 166°28.791'E, Nouméa, New Caledonia). Species identifications were
254 performed by combining morphological and genetic analyses following Vieira *et al.* (2014). Vouchers for each
255 species are kept at IRD herbarium (IRD10213, IRD10195, IRD10187, IRD10199). *Lobophora rosacea* has a
256 thin fan-shaped thallus, growing fixed by the basal part within coral branches like *Acropora* spp. *Lobophora*
257 *monticola* is also found associated to branching corals, and thalli grows partially or completely in contact with
258 them. *Lobophora sonderii* forms dense erected blades, mixed with other brown seaweeds in *Sargassum* beds.
259 Conversely, *L. obscura* has encrusting and thick leather-like thalli, strongly attached to dead corals or coral
260 rubbles (Fig. 5). Specimens (six for *L. rosacea*, *L. monticola* and *L. obscura* and five for *L. sonderii*) were placed
261 in separate ziplock plastic bags, immediately placed into ice and stored at -20 °C until sample grinding.

262

263 **5.2. Metabolite extractions**

264 Algae were freeze-dried and manually ground with liquid nitrogen in a mortar. Samples were then stored in
265 silicagel until chemical extractions. For each replicate, a mass of 250 mg was extracted 3 times with 5 mL of
266 MeOH/CH₂Cl₂(1:1) during 5 min in an ultrasonic bath. Supernatants were pooled and filtered from samples. The
267 extracts were concentrated to dryness in the presence of C18 silica powder (100 mg, Polygoprep 60-50,
268 Macherey-Nagel®) using a rotary evaporator, and the solid was then fractionated by Solid Phase Extraction (SPE,
269 Strata C18-E 500 mg/6 mL, Phenomenex®) by the successive elution of H₂O (6 mL), MeOH (6 mL) and CH₂Cl₂
270 (6 mL) after cartridge cleaning (6 mL MeOH/CH₂Cl₂) and conditioning (6 mL H₂O). The MeOH fractions were
271 then filtered on syringe filters (PTFE, 0.20 µm, Phenomenex®), dried in a speedvac and further used for
272 UHPLC-QToF and NMR analyses. The CH₂Cl₂ fractions were only analyzed by GC/MS.

273

274 5.3. Metabolomic analyses

275 5.3.1. NMR

276 Dry samples were dissolved in 0.5 mL CDCl₃. ¹H-NMR spectra were recorded on a cryoprobe-equipped 600
277 MHz Agilent spectrometer. The following parameters were used for data acquisition: 16 ppm spectral width, 1 s
278 relaxation delay with water pre-saturation (PS), number of scans 32, acquisition time 1.7 s, 16 K complex data
279 points, 90° pulse angle.

280 5.3.2 UHPLC-QToF

281 Metabolomic fingerprints were recorded on an UHPLC (Dionex Ultimate 3000, Thermo Scientific®) coupled to
282 an accurate mass spectrometer equipped with an ESI source (QqToF Impact II, Bruker Daltonics®). Metabolite
283 separation were performed on a C18 UHPLC column (Acclaim™ RSLC 120 C18 150 x 2.1 mm, 2.2 µm,
284 Thermo Scientific®) at 40 °C. The mobile phase consisted in a mix of H₂O + 0.1 % formic acid + 10 mM
285 ammonium formate (solvent A) and acetonitrile/H₂O (95:5) + 0.1 % formic acid + 10 mM ammonium formate
286 (solvent B). Injection volume was set to 3 µL and elution flow to 0.4 mL min⁻¹. The elution gradient was
287 programmed as follows: 40 % B during 2 min, increased up to 100 % B from 2 to 8 min, followed by an isocratic
288 step of 100% B during 4 min. The initial conditions were gradually recovered from 12 to 14 min, and hold 3 min
289 for column equilibration for a total runtime of 17 min. MS parameters were set as follows: nebulizer gas N₂ at 40
290 psig, gas temperature 300 °C, drying gas N₂ flow 4 L min⁻¹, capillary voltage 3500 V. Mass spectra were
291 acquired in positive ionization mode from 50 to 1,200 amu at 2 Hz. Auto-MS² spectra were acquired according
292 to the same conditions then previously. A quality control sample (QC) was prepared with 25 µL of each sample.
293 It was used to check MS shift over time and to normalize data according to injection order. The run started with
294 three blank injections, followed by 10 injections of the QC for mass spectrometer stabilization. Samples were
295 then randomly injected, inserting one QC every five samples. A final blank was injected to check any memory
296 effect of the compounds on the column.

297

298 5.3.3 GC-MS

299 CH₂Cl₂ fractions were analyzed on a gas chromatograph (7890B GC System - 7693 autosampler, Agilent
300 Technologies®) coupled to a mass selective detector (5977A MSD, Agilent Technologies®). Separation of
301 metabolites was performed on a HP-5MS 5% Phenyl-Methyl Silox column (30 m x 0.25 mm, 0.25 µm, Agilent
302 Technologies®) with helium as mobile phase. The run started at 40 °C for 5 min and increased by 10 °C min⁻¹ up

303 to 300°C for a total runtime of 31 min. A constant flow rate was set to 1 mL min⁻¹. A volume of 1 µL of each
304 sample was injected in splitless mode at 250°C. A solution with a mix of C8-C20 and C21-C40 alkanes (Fluka
305 Analytical) was also injected for the determination of compound retention index.

306

307 *5.3.4 Data treatment*

308 ¹H-NMR spectra were automatically Fourier-transformed and processed on MesReNova 11. Spectra baselines
309 were automatically corrected followed by the Whittaker smoother correction. An equal width bucketing of
310 0.001 ppm was applied between 0-8 ppm to finally obtain the data matrix. Data were auto-scaled and log-
311 transformed before statistical analyses.

312 LC-MS raw data files were first calibrated before converting them to netCDF files (centroid mode) using Bruker
313 Compass DataAnalysis 4.3. NetCDF files were processed using the package XCMS for R software (version
314 3.3.2, XCMS version 1.50.1). Optimized parameters for XCMS were used as follows: peak picking
315 (method= "centwave", peakwidth= c(2,20), ppm= 15, mzdif= 0.05, prefilter= c(0,0)), retention time correction
316 (method= "obiwarp"), matching peaks across samples (bw= 30, mzwid= 0.015, minfrac= 0.3) and filling in
317 missing peaks. The matrix was then cleaned according to blanks and pooled samples to remove analytical
318 variability. Molecular network based on MS² spectra were constructed with GNPS (Wang et al., 2016) and
319 managed under Cytoscape 3.5.0 (Shannon et al., 2003).

320 Agilent data files acquired from GC-MS analysis were exported into CDF files using MSD Chemstation
321 (F.01.001903, Agilent Technologies®). CDF files were then processed using the package eRah (version 1.0.5,
322 Domingo-Almenara et al., 2016) under R performing preprocessing, peak deconvolution (min.peak.width = 2.5,
323 min.peak.height=2500, noise.threshold=500, avoid.processing.mz=c(73,149,207)), peak alignment
324 (min.spectra.cor=0.90, max.time.dist=60, mz.range=40:500) and missing compound recovery (with presence
325 required in 3 samples at least). Compound annotation was performed manually by comparing mass spectra with
326 NIST 2011 database completed with the calculation of Kovàts' index (Van Den Dool and Kratz, 1963). The
327 matrix obtained was finally filtered according to the blank. Data from LC-MS and GC-MS were normalized by
328 log-transformation before statistical analyses.

329

330 **5.4. Statistical analyses**

331 Principal component analysis (PCA) was used to visualize the metabolome variation according to species (ade4
332 package for R). Powered Partial Least-Squares-Discriminant Analysis (PPLS-DA) identified the maximum
333 covariance between our data set and their class membership and permutational tests based on cross model
334 validation (MVA.test and pairwise.MVA.test) were used to test differences between groups (RVAideMemoire
335 package). Discriminating compounds were then identified according to the PPLS-DA loading plots (correlation
336 circles; RVAideMemoire package). Multiple Factor Analysis (MFA, variables scaled to unit variance) was used
337 to combine data obtained from LC-MS and NMR (FactoMineR and factoextrapackages for R). Kruskal-Wallis
338 tests were performed in MetaboAnalyst 3.0 and R (PMCMR package). Post-hoc Conover's test was done on R
339 software (PMCMR package). Venn diagram was constructed with Venny 2.1 (Oliveros 2007-2015).

340

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347

348 **Conflict of Interest:** The authors declare no conflicts of interest.

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523

524 **Figure and Table captions**

525

526 **Fig. 1.** Discriminant power of the three chemical approaches via unsupervised (Principal Component Analysis,
527 PCA, a, c, e) and supervised discriminant (Powered Partial Least-Squares-Discriminant Analysis, PPLS-DA, b,
528 d, f) analyses of *Lobophora* species metabolome analyzed by (a, b) NMR for MeOH fractions and (c, d) LC-MS
529 for MeOH fractions and (e, f) GC-MS for CH₂Cl₂ fractions (LO: *L. obscura* in red, LR: *L. rosacea* in orange,

530 LM: *L. monticola* in green and LS: *L. sonderii* in blue). CER = classification error rate with p-value after double
531 cross model validation.

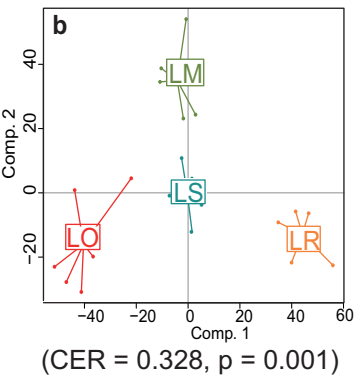
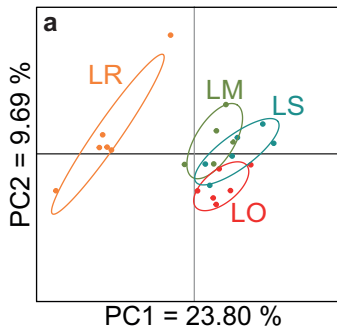
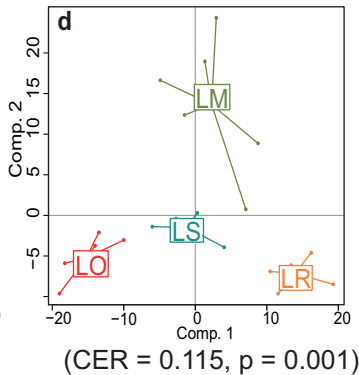
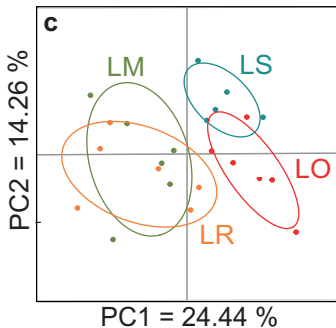
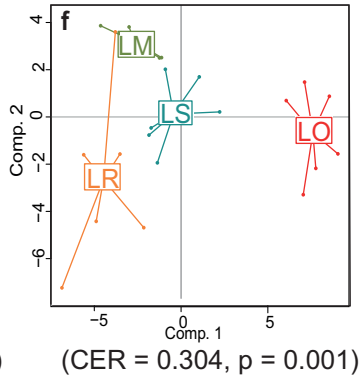
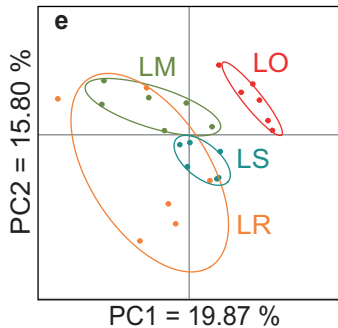
532 **Fig. 2.** Overlay of ¹H-NMR (600 MHz) spectra of the four *Lobophora* species (one representative sample per
533 species was chosen, the full overlay spectra is available in Fig. S3. *L. monticola* in red, *L. obscura* in green, *L.*
534 *rosacea* in blue and *L. sonderii* in purple). Regions of discriminating signals are highlighted by black rectangles.
535

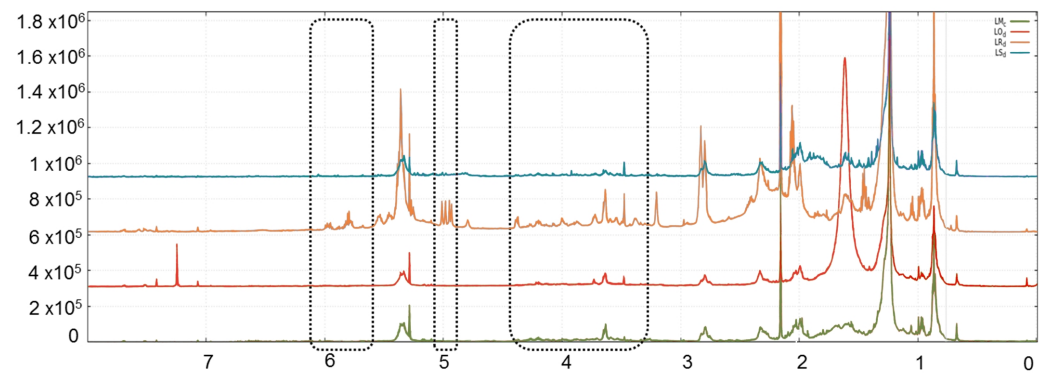
536 **Fig. 3.** Box plots of lobophorenols B and C (and chemical structure of lobophorenols A-C) among the four
537 *Lobophora* species and blank (log-transformed data, y-axis), detected by LC-MS, expressed as mean normalized
538 intensities ± SD (n = 3 for blank, n = 6 for LM, LO, LR and n = 5 for LS)(LM: *L. monticola* in green, LO: *L.*
539 *obscura* in red, LR: *L. rosacea* in orange and LS: *L. sonderii* in blue, x-axis). The statistical analyses were
540 performed using Kruskal-Wallis (KW) followed by post-hoc Conover's test. Letters indicate significant
541 differences between groups based on post-hoc pairwise comparisons (p < 0.05).

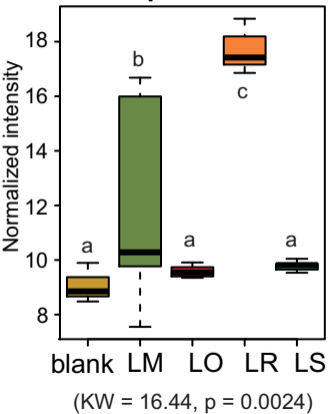
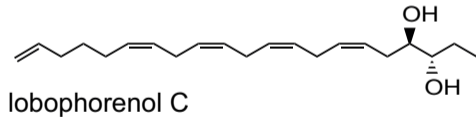
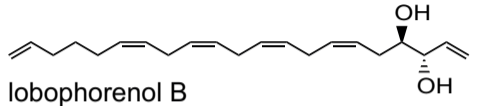
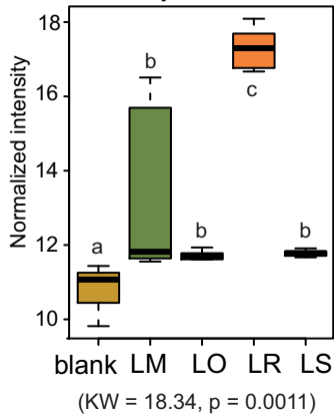
542 **Fig. 4.** Multiple Factor Analysis (MFA) obtained with LC-MS and NMR data from the MeOH fractions of
543 *Lobophora* species (LM: *L. monticola* in green, LO: *L. obscura* in red, LR: *L. rosacea* in orange, and LS: *L.*
544 *sonderii* in blue). Confidence level used to construct the ellipses = 0.95, variables scaled to unit variance.

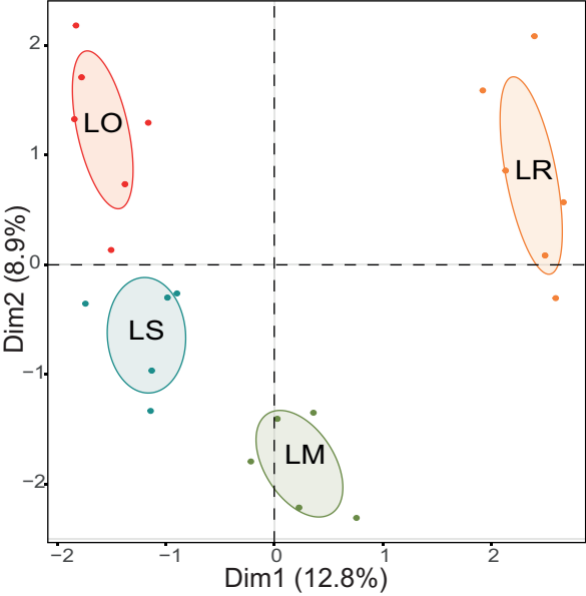
545 **Fig. 5.** Pictures of *Lobophora* species: (a) *L. rosacea*, (b) *L. sonderii*, (c) *L. obscura* and (d) *L. monticola*.
546 Arrows indicate algal thalli (algae were collected at Ricaudy for (a), (b), (c) and Sainte-Marie for (d); images by
547 G. Boussarie).

548 **Table 1.** Chemomarkers detected by GC-MS in the CH₂Cl₂ fraction of *Lobophora* species, annotated with NIST
549 2011 database (COMP. = compound, RI = Van Den Dool and Kratz Retention Index, EXP. = experimental, LIT.
550 = literature).
551

NMR**LC-MS****GC-MS**



lobophorenol B**lobophorenol C**



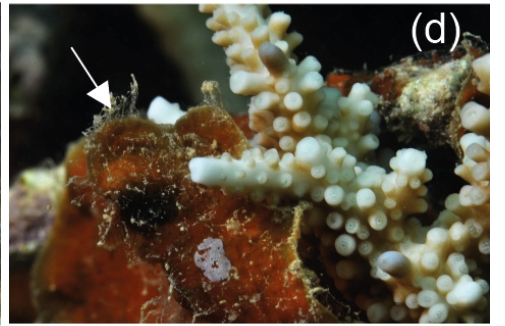
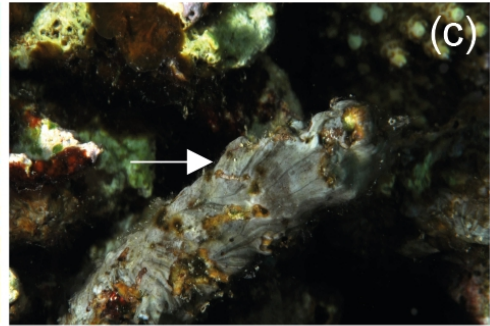
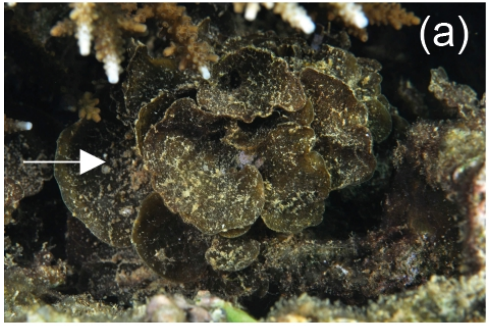


Table 1. Chemomarkers detected by GC-MS in the CH₂Cl₂ fraction of *Lobophora* species, annotated with NIST 2011 database (COMP. = compound, RI = Van Den Dool and Kratz Retention Index, EXP. = experimental, LIT. = literature).

COMP.	MOLECULAR NAME	CHEMICAL FAMILY	CAS NUMBER	RAW FORMULA	% MATCH NIST 2011	LIT. RI	EXP. RI
M5	2-pentenoic acid	carboxylic acid	626-98-2	C ₅ H ₈ O ₂	94	873	921
M18	maleimide, 2-methyl-3-vinyl	amide	21494-57-5	C ₇ H ₇ NO ₂	91	1261	1262
M38	methyl stearate	ester	112-61-8	C ₁₉ H ₃₈ O ₂	98	2130	2129
M48	hexanoic acid, 2-ethyl-, hexadecyl ester	ester	59130-69-7	C ₂₄ H ₄₈ O ₂	64	-	2468

SUPPLEMENTARY INFORMATION

Metabolomic variability of four macroalgal species of the genus *Lobophora* using diverse approaches

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FIGURES

- Fig. S1.** Chemical structure of lobophorenols A-C.3
- Fig. S2.** Kruskal-Wallis test loading plots with bins varying among species in red ($p < 0.05$). Data were obtained with NMR.3
- Fig. S3.** Overlay of $^1\text{H-NMR}$ (600 MHz) spectra of the four *Lobophora* species (*L. monticola* in red, *L. obscura* in green, *L. rosacea* in blue and *L. sonderii* in purple). Regions of discriminating signals are highlighted by black rectangles.3
- Fig. S4.** Venn diagram test on *Lobophora* MeOH fractions analyzed by LC-MS (LO: *L. obscura*, LR: *L. rosacea*, LM: *L. monticola* and LS: *L. sonderii*). Minimal threshold was selected for each species when the ion intensity detected by LC-MS become very low and closed to the noise.4
- Fig. S5.** Box plots of the chemomarkers annotated with NIST 2011 among the four *Lobophora* species (log-transformed data), detected by GC-MS, expressed as mean normalized intensities \pm SD ($n = 6$ for each species) (LM: *L. monticola* in green, LO: *L. obscura* in red, LR: *L. rosacea* in orange, and LS: *L. sonderii* in blue). The statistical analyses were performed using Kruskal-Wallis (KW) followed by post-hoc Conover's test. Letters indicate distinct groupings based on post-hoc pairwise comparisons among groups for each compound ($p < 0.05$)4
- Fig. S6.** *Lobophora* species tree reconstructed with BEAST using the mitochondrial marker *cox3* and the chloroplast markers *psbA* and *rbcl* (adapted from Vieira et al. 2016, 2017) (LO: *L. obscura*, LR: *L. rosacea*, LM: *L. monticola* and LS: *L. sonderii*).5
- Fig. S7.** Example of chromatogram obtained by GC-MS on the CH_2Cl_2 fraction of *Lobophora rosacea*.6
- Fig. S8.** Example of chromatogram obtained by LC-MS on the MeOH fraction of *Lobophora rosacea* with the elution peaks corresponding to the lobophorenols B and C.7
- Fig. S9.** Molecular network on MS^2 spectra managed under Cytoscape 3.5.0, with parent mass label. Lobophorenol B (m/z 334.272) and lobophorenols C (m/z 336.287) are in red.8

TABLES

- Table S1.** Post-hoc permutational pairwise test based on crossed model validation for metabotype differentiation according to species by NMR, LC-MS or GC-MS (999 permutations, p-value adjustment method: fdr). Significant p-value ($p < 0.05$) are in bold.9
- Table S2.** Selection of the most significant regions in the spectra varying among *Lobophora* species (from Kruskal Wallis test, with p -value < 0.05). Characteristic signals (ppm) of lobophorenols A, B and C are also assigned to the corresponding chemical shift range.9
- Table S3** *Lobophora* ions responsible for the difference according to species after LC-MS analysis. The mSigma (mS) value is a measure for the goodness of fit between experimental mass and isotopic pattern with theoretical ones: lower is the mS, better is the annotation.9

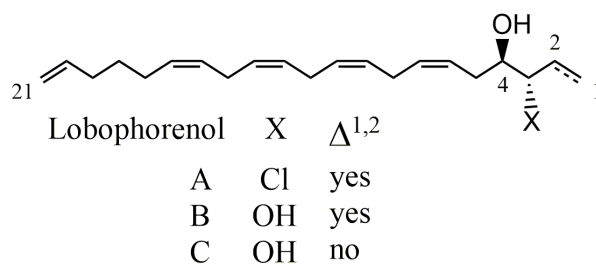


Fig. S1. Chemical structure of lobophorenols A-C.

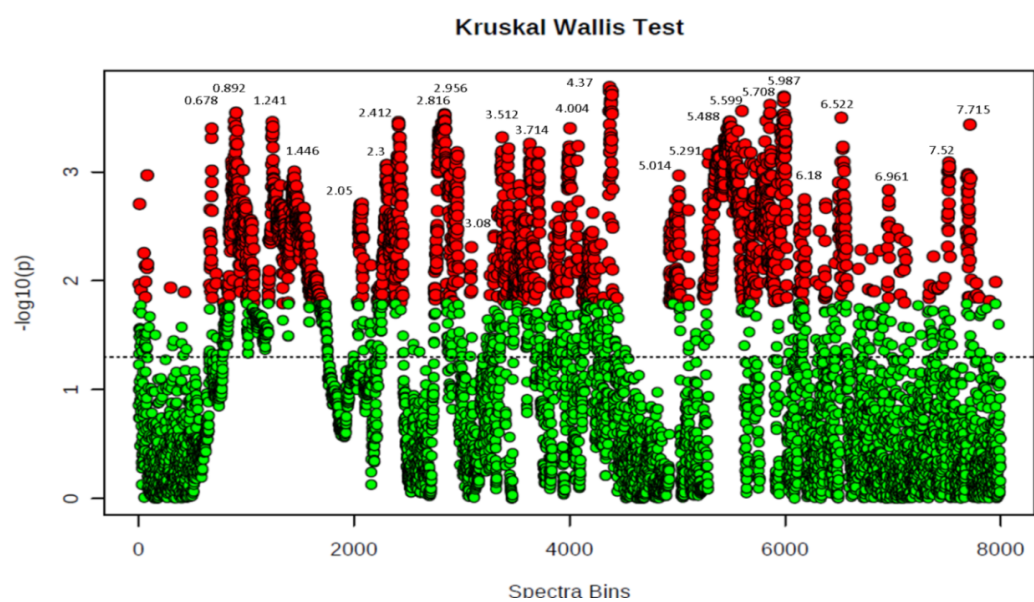


Fig. S2. Kruskal-Wallis test loading plots with bins varying among species in red ($p < 0.05$). Data were obtained with NMR.

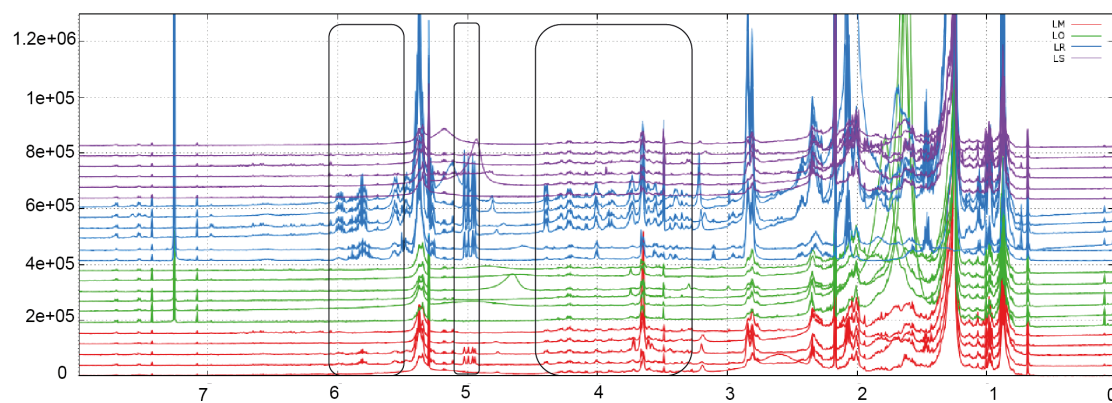


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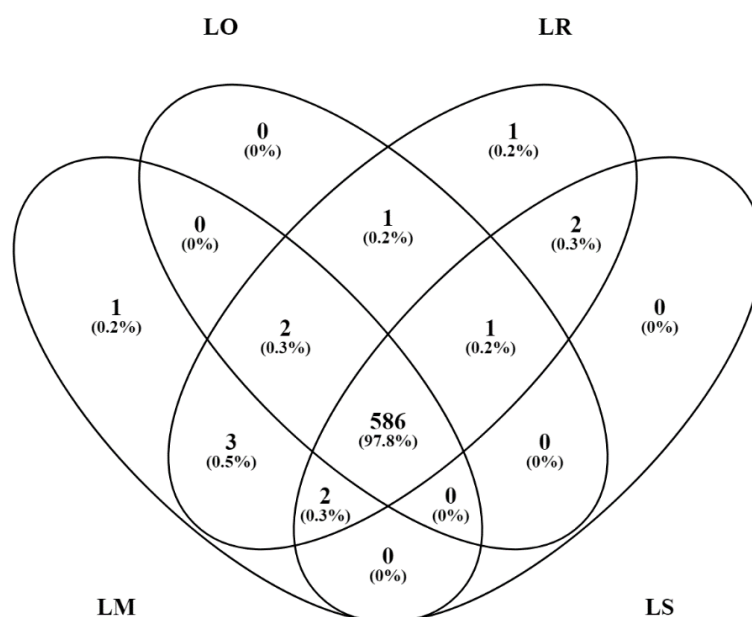


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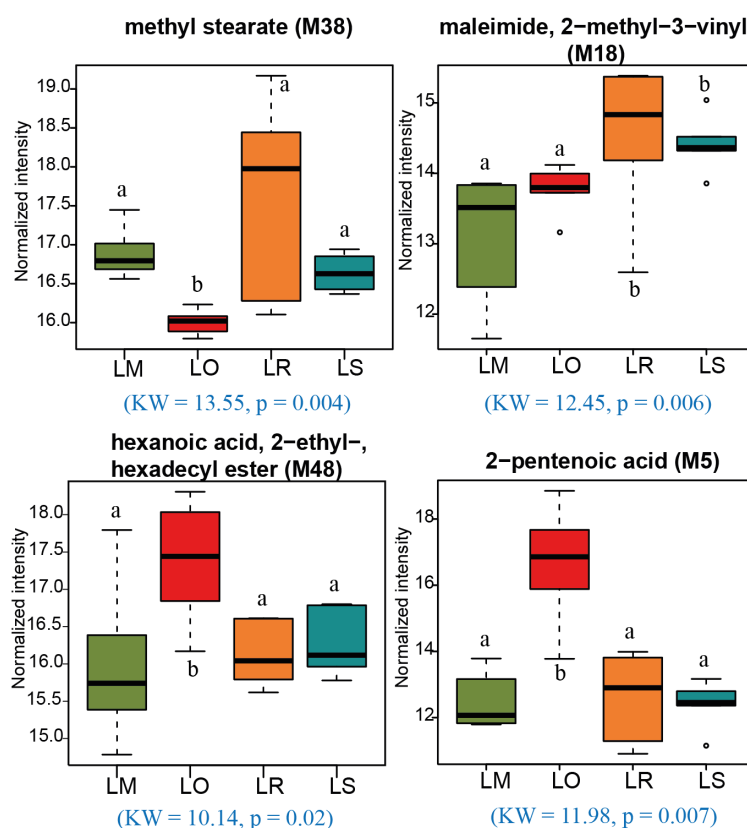


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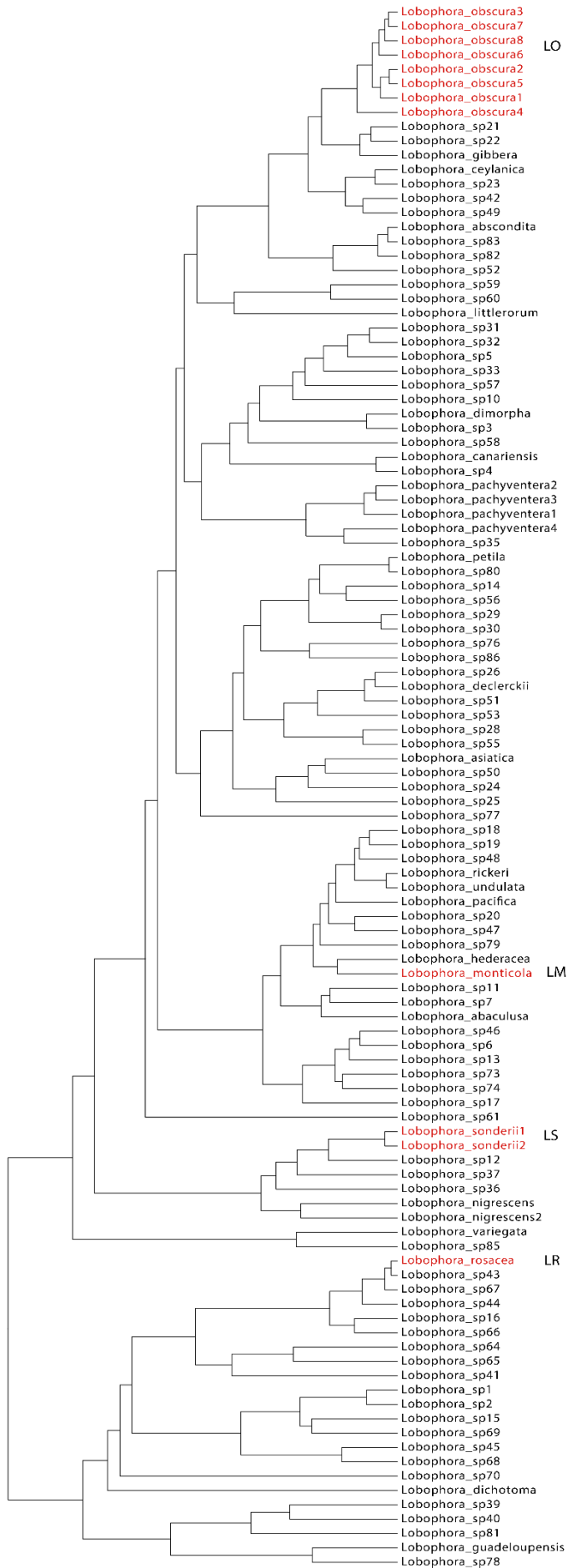


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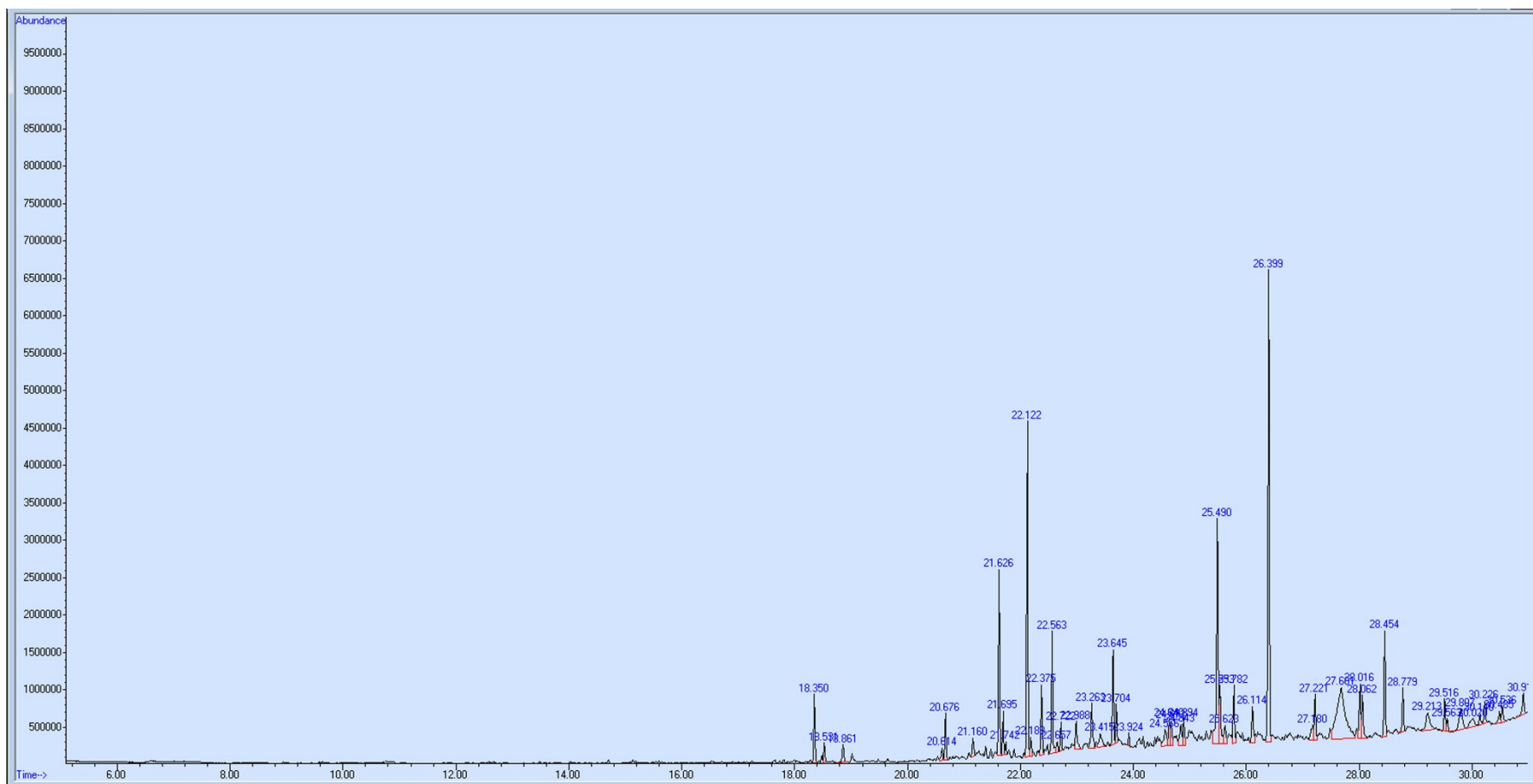


Fig. S7. Example of chromatogram obtained by GC-MS on the CH₂Cl₂ fraction of *Lobophora rosacea*.

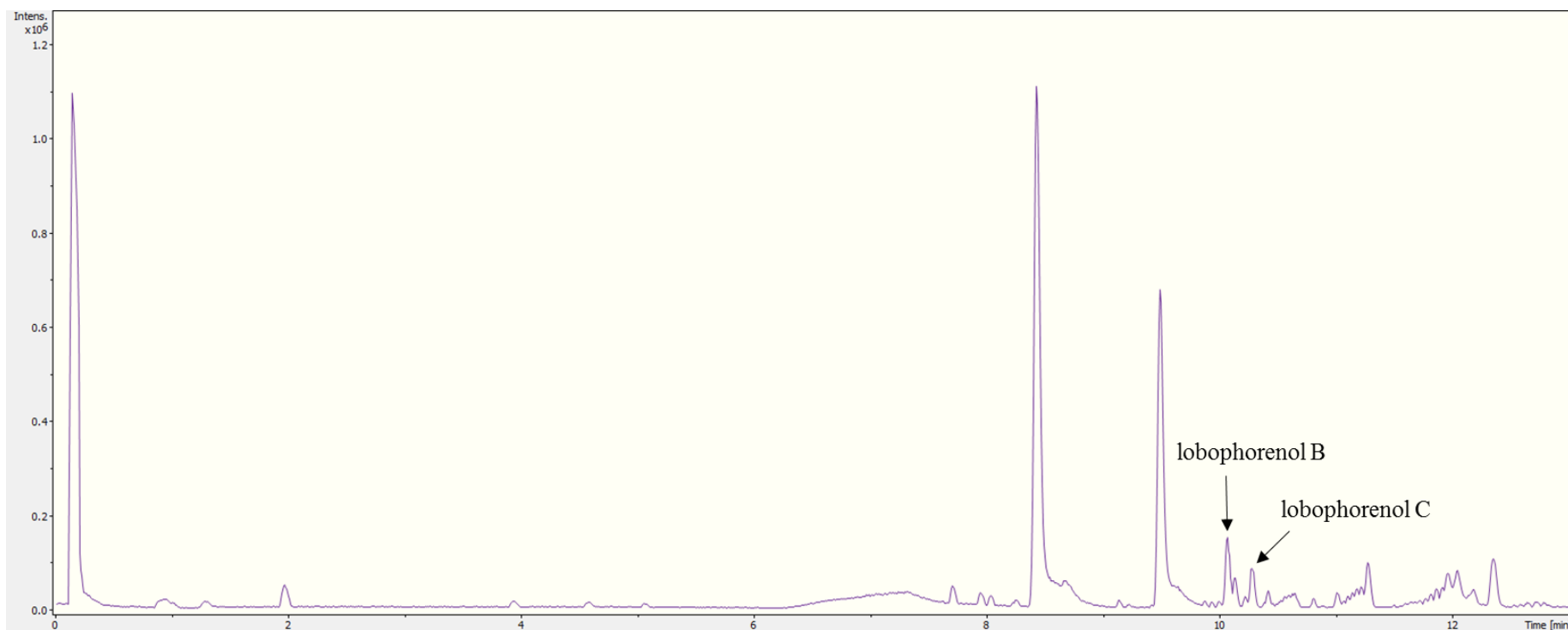


Fig. S8. Example of chromatogram obtained by LC-MS on the MeOH fraction of *Lobophora rosacea* with the elution peaks corresponding to the lobophorenols B and C.

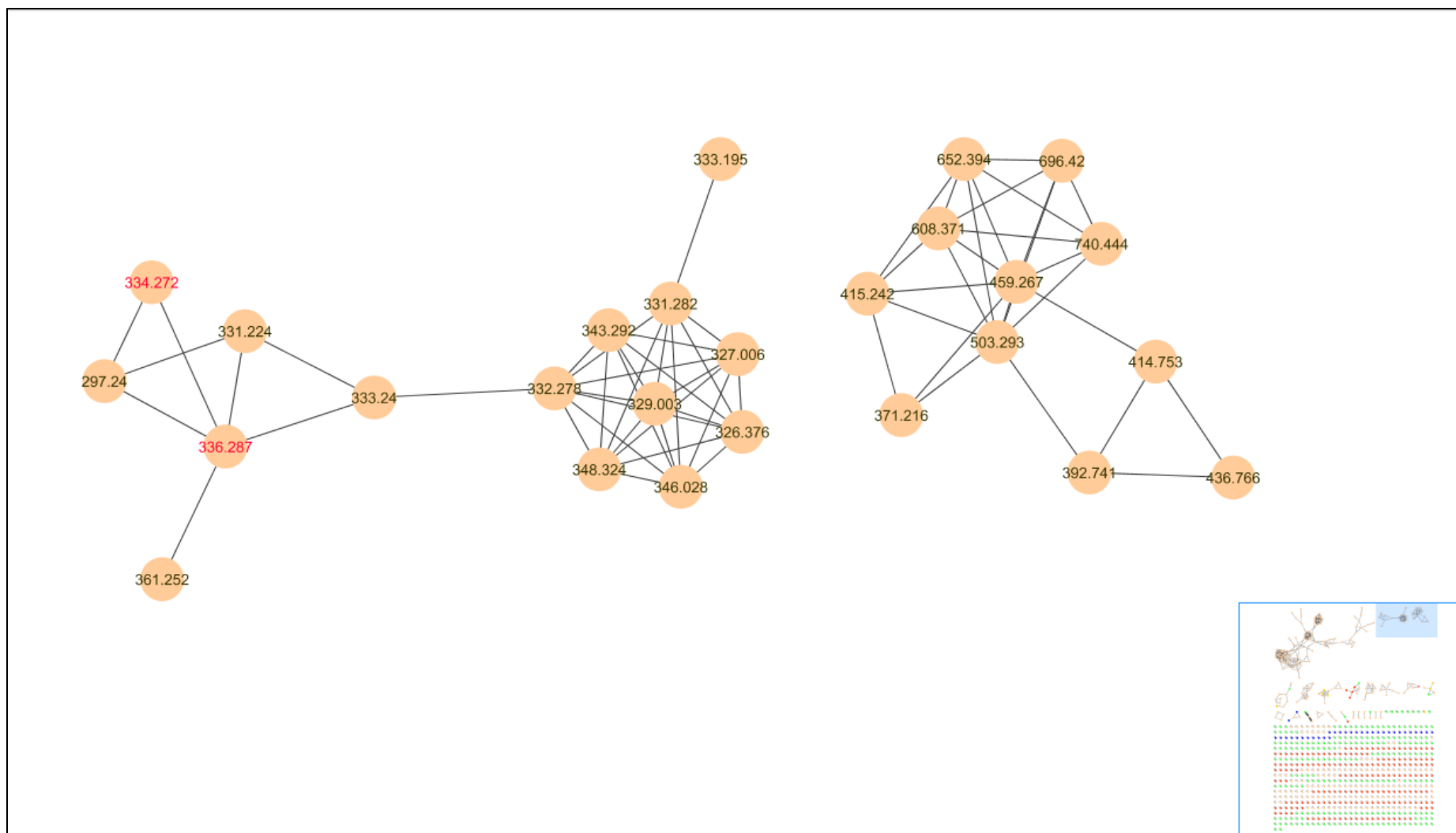


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Table S1. Post-hoc permutational pairwise test based on crossed model validation for metabotype differentiation according to species by NMR, LC-MS or GC-MS (999 permutations, p-value adjustment method: fdr). Significant p-values ($p < 0.05$) are in bold.

NMR			LC-MS			GC-MS					
	LM	LO	LR		LM	LO	LR		LM	LO	LR
LO	0.018	-	-	LO	0.006	-	-	LO	0.006	-	-
LR	0.022	0.006	-	LR	0.044	0.006	-	LR	0.431	0.008	-
LS	0.276	0.276	0.018	LS	0.006	0.018	0.006	LS	0.012	0.008	0.018

Table S2. Selection of the most significant regions in the spectra varying among *Lobophora* species (from Kruskal-Wallis test, with p-value < 0.05). Characteristic signals (ppm) of lobophorenols A, B and C are also assigned to the corresponding chemical shift range.

Max ppm area	Range ppm area	Characteristic signals (ppm)
0.678	0.662 - 0.68	-
0.892	0.823 - 0.997	Lobophorenol C: 0.97
1.214	1.237 - 1.253	-
1.446	1.407 - 1.501	Lobophorenol C: 1.47 Lobophorenol A, B & C: 1.46
2.05	2.035 - 2.077	Lobophorenol B: 2.06, A & C: 2.07
2.3	2.08 - 2.386	Lobophorenol A: 2.10/2.25, B: 2.14, C: 2.24
2.412	2.318 - 2.433	Lobophorenol B & C: 2.36
2.816	2.747 - 2.836	Lobophorenol A, B & C: 2.82
2.956	2.85 - 3.08	Lobophorenol A: 2.86/2.87
3.374	3.240 - 3.442	Lobophorenol C: 3.32
3.512	3.365 - 3.595	Lobophorenol B: 3.48, C: 3.46
3.714	3.536 - 3.721	Lobophorenol A: 3.70
4.004	3.854 - 4.302	Lobophorenol B: 3.94
4.37	4.103 - 4.398	Lobophorenol A: 4.38
5.014	4.927 - 5.102	Lobophorenol A, B & C: 4.94 and 5.00
5.291	5.244 - 5.397	Lobophorenol A: 5.34, A, B & C: 5.37 and 5.38
5.488	5.398 - 5.667	Lobophorenol A: 5.48/5.49, B & C: 5.45 and Lobophorenol B: 5.51, C: 5.52
5.599	5.399 - 5.74	-
5.708	5.589 - 5.860	Lobophorenol B & C: 5.82
5.987	5.837 - 6.013	Lobophorenol B: 5.92
6.18	6.009 - 6.377	Lobophorenol A: 6.02
6.522	6.365 - 6.534	-
6.961	6.682 - 7.12	-
7.52	7.376 - 7.529	-
7.715	7.684 - 7.779	-

Table S3. *Lobophora* ions responsible for the difference according to species after LC-MS analysis. The mSigma (mS) value is a measure for the goodness of fit between experimental mass and isotopic pattern with theoretical ones: lower is the mS, better is the annotation.

m/z	rt	Ion assignment	Ion formula	error (ppm)	mS
334.2741	606	[M + NH ₄ ⁺]	C ₂₁ H ₃₆ NO ₂	-2.5	9.4
336.2898	619	[M + NH ₄ ⁺]	C ₂₁ H ₃₈ NO ₂	-1.5	21.5