

1 Epicuticular wax lipid composition of endemic European *Betula*
2 species and its application to chemotaxonomy and paleobotany

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25 **Abstract**

26 Plants, in particular trees with specific habitat demands are excellent indicators of climate state.
27 Vegetation successions in subrecent and deep geologic time is recorded in fossil macro-remains
28 or pollen accumulating in geological archives like limnic and marine sediments, peat bogs and
29 mires. Birch trees in Europe form a major part in plant successions and constitute the dwarf
30 species *Betula nana* and *Betula humilis* representing cold-adapted habitats or climates and two
31 tree birches, *Betula pubescens* and *Betula pendula* characteristic for temperate habitats or
32 climates. These birch species exhibit highly similar pollen shape and size, preventing their
33 unambiguous application as paleoclimate/paleovegetation proxies. We here present a
34 chemotaxonomic differentiation of the four European birch species based on their epicuticular
35 wax lipids. The dominating lipid classes in epicuticular birch waxes were found to be n-alkanes
36 (in the range of *n*-C₂₃ to *n*-C₃₃), straight-chain primary alcohols and fatty acids (in the range of
37 *n*-C₂₀ to *n*-C₃₂), and long-chain wax ester (in the range of *n*-C₃₈ to *n*-C₄₆) in variable amounts
38 and distributions. When preserved in geological archives these lipids may serve in
39 paleovegetation/paleoclimate reconstruction. Long-chain wax esters are susceptible to
40 hydrolysis and upon diagenesis the release of ester-bound alcohols and fatty acids may modify
41 the distribution pattern of the corresponding primary free lipids. Quantitative analysis of the
42 hydrolyzable wax ester proportion revealed primary distribution patterns of birch lipids not to
43 change substantially upon release of bound analogues. The specific composition and
44 abundance of epicuticular wax lipids facilitates unambiguous chemotaxonomic separation of
45 the four European birch species. Wax lipid-based discrimination in field application, however,
46 is complicated by mixing of alkyl lipids derived from different birch species and contribution
47 of wax lipids from other plants. In cases, where palynology indicates a high contribution of
48 *Betula* species to European vegetation associations, wax lipids may serve for differentiation of
49 the species contributing.

50

51 **Introduction**

52 Environmental demands, in particular climate, govern the present-day habitat and distribution
53 of trees, which in turn facilitates determination of climate regimes in the contemporaneous as
54 well as in the fossil domain. Taxonomy of present-day trees is based on anatomical,
55 morphological, genetic and biochemical studies, whereby such features in the fossil record are
56 best preserved in pollen distributions, due to a higher recalcitrance of pollen versus other plant
57 organs, e.g. leaves and rare findings of other macro-remains, e.g. fruits. Under special
58 conditions of preservation though fossil remains can be found in sediments dating back to the
59 Eocene [1]. Paleovegetation and related paleoclimate reconstruction thus heavily relies on
60 palynology, accompanied by analysis of macro-remains when present. Genetic [2–4] and
61 biochemical approaches [5–8] are rare, except for molecular and isotopic investigation of
62 leaf/needle wax, the latter rarely conducted on the species level.

63 Birches (*Betula* L., Betulaceae) are common broadleaf trees and shrubs occurring in diverse
64 habitats of the boreal and the cold-temperate zones of the Northern Hemisphere [9,10]. Ranging
65 from temperate zones in Northern America over Eurasia to East Asia and the circumpolar
66 regions, birches populate different habitats including forests, swamps, tundra and mountainous
67 terrains [11]. The number of species belonging to the genus *Betula* and their relationships is
68 still under debate ranging from 30 to 60 different taxa within 4 to 6 subgenera [3,12–15]. Of
69 these, four *Betula* species are endemic to Europe. The two tree birches, *Betula pubescens*
70 (downy birch) and *Betula pendula* (silver birch) occur throughout most of Europe, whereby
71 *Betula pubescens* has a more northerly and easterly distribution, while *Betula pendula* reaches
72 more southern regions such as the Iberian Peninsula, Italy and Northern Greece [10,15]. Two
73 dwarf/shrubby birch species, namely *Betula nana* (arctic dwarf birch) and *Betula humilis*

74 (dwarf birch) thrive in Europe as well but are confined to a much smaller growth range. *Betula*
75 *nana* is preferentially located at higher elevations like the Alps and Carpathian Mountains or
76 in perennial colder regions like Northern Europe from Iceland over northern Scotland to
77 Scandinavia [10,15]. *Betula humilis* has a wide distribution from western Germany to eastern
78 Siberia and Korea, but its occurrence is very scattered with only a few habitats left in Central
79 Europe [15]. As an important component of plant succession found in highly contrasting
80 climate and growth regimes, these *Betulaceae* and their respective habitat demands are
81 sensitive (paleo)climate and (paleo)environmental indicators, provided that they can be
82 taxonomically differentiated.

83 Recent birch species can be distinguished by their leaf and catkin/fruit shape [16,17] even when
84 fossilized in sediments, whereby their evolutionary relation and phylogeny is difficult to assess
85 due to intensive hybridization in nature. Extensive hybridization and introgression has been
86 investigated not only within a subgenus but also across *Betula* species of different subgenera
87 [18,19]. Precise classification is complicated by an spatial overlap of natural habitats for
88 European birch species enabling natural hybridization [20]. The identification of birch remains
89 in geological archives for paleovegetation reconstruction is limited by a common lack of well-
90 preserved leaves or catkins occurring in statistically relevant quantities. However, the
91 identification of birch vegetation over time is of great interest, especially during the Late
92 Glacial and Early Holocene (approx. 15,000 – 9,000 a BP), to understand early colonization
93 and forest/shrub expansion in deglaciated landscapes as well as the adaptation of vegetation to
94 climate variability and perturbation. Macrofossil findings revealed a dominance of tree birches,
95 like *Betula pubescens*, during temperate phases, whereas upon glacial stages *Betula nana* was
96 the most abundant birch species, serving as a tundra indicator [21].

97 Most commonly, paleovegetation reconstruction in sedimentary archives like lakes, peats and
98 bogs is based on palynological approaches, since macro remains are mostly absent [22–30].

99 However, differentiation of birch species by pollen is challenging, due to similar morphological
100 traits, e.g. shape, diameter and depth of pores in pollen within the genus *Betula*, which leads to
101 overlap in pollen-size distribution [31–35]. In addition, pollen morphology can also be effected
102 by the chemicals used upon preparation, pollen maturity, type of mounting medium, but also a
103 latitudinal and altitudinal effect cannot be excluded (reviewed in 32). In this study we present
104 the epicuticular leaf wax composition as a complementary proxy for recognition and
105 differentiation of the four European birches, with the potential to employ the wax distribution
106 patterns in paleovegetation reconstruction.

107 Terrestrial plant leaves are covered by a hydrophobic barrier to protect against the loss of water
108 due to evaporation, mechanical damage, ultraviolet radiation and bacterial or fungal pathogens
109 [36–39]. This barrier consists of an epicuticular wax layer, composed of long-chain alkyl
110 compounds including amongst others *n*-alkanes, *n*-alcohols, *n*-alkanoic acids, *n*-alkyl esters, *n*-
111 aldehydes, *n*-ketones and others [37,40]. Their composition is highly variable in quality and
112 quantity across plant species and therefore has high chemotaxonomic potential [41,42,51,43–
113 50]. *n*-Alkanes with carbon chain-length between C₂₅ and C₃₅ carbon atoms are associated with
114 higher plants with a strong odd-over-even predominance (expressed as the carbon preference
115 index - CPI), while their shorter chain homologues (<C₂₀), especially *n*C₁₇, are mainly found
116 in aquatic microorganisms [52,53]. Intermediate chain-length *n*-alkanes with C₂₃ and C₂₅
117 predominance are primarily found in aquatic macrophytes and in mosses of the genus
118 *Sphagnum* [49,54,55].

119 Both, *n*-alcohols and *n*-alkanoic acids in contrast to the *n*-alkanes hold an even-over-odd
120 predominance in carbon chain-lengths, which typically ranges from C₂₀ to C₃₂ [37]. Alkyl esters
121 consist of even-numbered *n*-alkanoic acids, which are esterified to even-numbered *n*-alcohols,
122 generating long-chain aliphatic compounds with on average 38 to 52 carbon atoms [56].
123 Among these lipid classes, the *n*-alkanes abundances are most frequently reported in vegetation

124 reconstruction, since they are very robust against alteration processes due to the lack of
125 functional groups. *Betula*-derived *n*-alkanes can be found in a variety of geological archives
126 including peats, soils, limnic and marine sediments of different ages ranging from modern
127 times up to several million years [45,57–59] and are easily extracted from sediments and leaves
128 by geochemical methods [37]. Therefore, most studies conducted to investigate
129 paleovegetation history employing leaf wax lipids are based on *n*-alkanes [60]. However, the
130 use of several lipid classes instead of a single will increase the discriminative power and
131 representativeness of the wax lipid composition. Studies involving *n*-alkanoic acids, *n*-alcohols
132 or *n*-alkyl ester for paleovegetation reconstruction are highly underrepresented [61,62], as these
133 lipids are usually not reported from modern plant homologues and their degree of preservation
134 in natural archives may vary [63]. Following incorporation into soil or sediment, wax esters
135 can be hydrolysed, releasing free *n*-alcohols and *n*-alkanoic acids. These in turn can be
136 converted into *n*-alkanes by decarboxylation and reduction, respectively [60]. Diagenetic fate
137 of functionalized lipids may vary depending on various factors such as oxygen availability and
138 pH affecting microbial reworking, but the potential of functionalized lipid classes in
139 paleobotany has been proven for sediments of up to Miocene age [63,64].

140

141 **Material and Methods**

142 **Leaf samples and collection**

143 Fresh leaf samples were collected in the Botanical Garden at Kiel University in September
144 2017. Three leaves from each birch species were taken from branches at different sites of the
145 tree from a height between 1 and 3 m. To avoid contaminations during sampling gloves were
146 worn and leaves were stored in glass container or aluminium foil until extraction. Subsequently
147 to sampling leaves were dried at 35°C in an oven for 48h. The mean annual air temperature for

148 Kiel-Holtenau is about 9.34°C and the total annual precipitation is about 744 mm (1987-2018)
149 (DWD, 2019)

150

151 **Lipid extraction**

152 Lipids were extracted by immersing a single leaf sample (0.02 – 0.27 g) for 60 s in a 30-50 ml
153 hexane/dichloromethane solution (1:1 v/v). The resulting solution was filtered through NaSO₄,
154 evaporated under vacuum at 50°C in a Büchi solvent evaporator and transferred into pre-
155 weighted vials. Per species three leaves were extracted to calculate standard deviation of lipid
156 concentration and composition. Prior to analyses, an aliquot of the total lipid extract (TLE) was
157 treated with 35 µl *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 5 µl pyridine at
158 70°C for 1 h to convert the *n*-alkanoic acids and *n*-alcohols to their corresponding trimethylsilyl
159 (TMS) derivatives. 10 µg of perdeuterated tetracosane (C₂₄), octadecanol (C₁₈), and eicosanoic
160 acid (C₂₀) were added as internal standard for quantification. All samples were analysed by gas
161 chromatography-mass spectrometry (GC/MS).

162

163 **Gas chromatography-mass spectrometry (GC-MS)**

164 The wax lipids were analysed using an Agilent 7890A (GC) equipped with an Agilent DB-5
165 column (30m x 0.25mm x 0.25µm) coupled to an Agilent 5975 B (MS). The oven program
166 started at 60°C for 4 min, followed by a ramp to 140°C at 10°C/min and subsequently to 325°C
167 at 3°C, followed by an isothermal period of 45 min. The MS operated with a scanning mass
168 range of *m/z* 50-850 at an ionization energy of 70 eV. All compounds were identified by using
169 authentic standards, NIST 14 library or their specific fragmentation pattern.

170

171 Leaf wax characteristic calculations

172 The *n*-alkane content of plant species was calculated as µg/g dry weight (d.w.) of leaf based on
173 mean values of triplicate analysis with standard deviation (Fig. 1).

174 Average chain length (ACL) for *n*-alkanes with 23 to 33 carbon atoms was calculated as:

$$175 \quad ACL = \frac{(23 \times nC_{23} + 25 \times nC_{25} + 27 \times nC_{27} + 29 \times nC_{29} + 31 \times nC_{31} + 33 \times nC_{33})}{(nC_{23} + nC_{25} + nC_{27} + nC_{29} + nC_{31} + nC_{33})}$$

176 with C_n as relative abundance of *n*-alkanes with the chain length *n* [65]. This proxy is used as
177 weighted mean of *n*-alkane carbon chain length, which supposed to vary with climate. The
178 carbon preference index (CPI) outlines the relative abundance of odd-over-even carbon chain
179 lengths, whereby values >1 indicate a predominance of odd carbon chain lengths homologues.
180 CPI values for *n*-alkanes with 24 to 34 carbon atoms were calculated according to:

$$181 \quad CPI = 0.5 \times \left[\frac{(nC_{25} + nC_{27} + nC_{29} + nC_{31} + nC_{33})}{(nC_{26} + nC_{28} + nC_{30} + nC_{32} + nC_{34})} \right. \\ 182 \quad \left. + \frac{(nC_{25} + nC_{27} + nC_{29} + nC_{31} + nC_{33})}{(nC_{24} + nC_{26} + nC_{28} + nC_{30} + nC_{32})} \right]$$

183

184 Wax ester quantification

185 For total wax ester quantification, the respective wax ester peaks in the GC-MS chromatograms
186 were integrated and quantified against an internal standard (deuterated tetracosane). For a wax
187 ester of the type RCOOR' the diagnostic fatty acid ion is $RCOOH_2^+$, indicative for the alkanolic
188 acid chain length. R'-1⁺ derives from the corresponding alcohol moiety, however, its intensity
189 is low and difficult to detect. Therefore, the diagnostic acid fragments ($RCOOH_2^+$) of peaks
190 containing co-eluting alkyl esters of identical total mass but variable combinations of alcohol
191 and alkanolic acid moieties were integrated to determine the percentage of the respective isomer
192 contribution. Multiplication of isomer percentages by analogue abundances led to the

193 proportion of the esterified acids. The proportional amount of esterified alcohol was obtained
194 by subtracting the esterified acid from the corresponding total wax ester homologue.

195

196 **Results and discussion**

197 **Alkyl lipid distribution of plants from Botanical Garden of Kiel**

198 **University**

199 The focus of this study lies on the epicuticular wax composition of the four birches endemic to
200 Europe. The leaf wax compound classes *n*-alkanes, *n*-alcohols, *n*-alkanoic acid and *n*-alkyl
201 ester were present in all species at variable composition distributions (S1). Epicuticular alkyl
202 lipid abundances are reported as $\mu\text{g/g}$ dry weight (d.w.) of leaf.

203 The lowest total amount of epicuticular waxes was observed in the arctic dwarf birch *B. nana*
204 with $538.3 \mu\text{g/g}$ d.w., followed by *B. pendula* with $1293.8 \mu\text{g/g}$ d.w., $3131.7 \mu\text{g/g}$ d.w. in *B.*
205 *pubescens* and *B. humilis* with $4187.9 \mu\text{g/g}$ d.w..

206

207 ***n*-alkane of *Betula* epicuticular wax**

208 The carbon atom chain-lengths of *n*-alkanes varied from $n\text{C}_{23}$ to $n\text{C}_{33}$ and maximized either at
209 $n\text{C}_{27}$ or $n\text{C}_{31}$. A predominance of $n\text{C}_{27}$ was noted for *B. humilis* with $413.2 \pm 91.1 \mu\text{g/g}$ d.w.. In
210 relative proportion, $n\text{C}_{27}$ was the dominant *n*-alkane homologue in *B. nana*, though it occurred
211 in minor absolute concentrations ($7.8 \pm 2.5 \mu\text{g/g}$ d.w.) only. The *n*-alkanes of two tree birches,
212 *B. pubescens* and *B. pendula*, maximised at $n\text{C}_{31}$ with 799.0 ± 133.3 and $183.0 \pm 103.8 \mu\text{g/g}$ d.w.,
213 respectively, whereby the latter species additionally contained large proportions of $n\text{C}_{25}$ and
214 $n\text{C}_{27}$ alkanes. The average chain-lengths for odd-carbon-numbered *n*-alkanes in the range from
215 $n\text{C}_{23}$ to $n\text{C}_{33}$ (ACL₂₃₋₃₃) varied from 26.7 in *B. humilis* to 29.7 in *B. pubescens*, while *B. pendula*

216 und *B. nana* had intermediate ACL values of 28.0 and 28.5, respectively. As expected, in the
217 recent leaves a typical odd-over-even predominance was detected, which is expressed in high
218 CPI values. Highest CPI values were found in *B. pendula* averaging 41.3, followed by *B.*
219 *pubescens* with 36.4 and *B. nana* with 11.1. The CPI for *B. humilis* could not be calculated due
220 to the lack of even-numbered *n*-alkanes. The *n*-alkane CPI values of the three species were
221 high, indicating that there was no significant contamination by diagenetic or petroleum-derived
222 *n*-alkanes.

223 The birch trees examined in this study grew under identical environmental conditions in the
224 Botanical Gardens of Kiel University, thus a possible influence of climate or soil condition on
225 the wax lipid distribution should be identical for each species. The *n*-alkane compositions in
226 all four *Betula* species exhibited a dominance of longer-chain lengths homologues (nC_{27} , nC_{29} ,
227 nC_{31}) with a mean ACL of ca. 28, as typical of deciduous trees [49]. However, some species
228 had only one dominant homologue (*B. pubescens*, *B. humilis*), whilst others had a more
229 bimodal distribution with two dominant homologues (*B. nana*, *B. pendula*) (Fig. 1). The ACL
230 has been used in geological archives as a climate and plant species indicator. For example, it
231 has been recognized that higher ACL values correlated with higher temperatures in sediments
232 of Lake Malawi (east Africa), potentially as protection against heat to increase the melting
233 point of epicuticular waxes [66]. However, Diefendorf et al. 2017 in their compilation study
234 could not confirm a significant temperature control on ACL neither in C_3 woody plants nor in
235 C_3/C_4 grasses. Higher ACL values, due to a higher proportion of nC_{31} , nC_{33} and partially nC_{35} ,
236 were observed in C_4 grasses (*poaceae*) of arid zones in Africa, which distinguished them from
237 C_3 species from Peru and Australia [67]. A correlation between the preferred habitat of the four
238 birches from our study (arctic/alpine vs. temperate zone) and ACL is not noticeable, since
239 especially the two cold tolerant dwarf birches showed markedly different *n*-alkane
240 distributions. However, the measured ACL values were in accordance with the expected values,

241 since no extended long-chain *n*-alkanes with 33 or even 35 carbon atoms, typically for arid
242 grasses, were found.

243

244 **Fig 1: Distribution of *n*-alkanes, *n*-alcohols, *n*-alkanoic acids and *n*-alkyl esters ($\mu\text{g/g}$ dry**
245 **leaf) from epicuticular waxes of four European birch species collected the Botanical**
246 **Garden of Kiel (Germany). Note the different scale of y-axis for each row.**

247

248

249 ***n*-alcohols of *Betula* epicuticular wax**

250 As with the *n*-alkane distribution, a typical terrestrial higher plant pattern was observed,
251 yielding a strong even-over-odd dominance in carbon chain-lengths [68]. The *n*-alcohol chain-
252 lengths ranged from $n\text{C}_{16}$ to $n\text{C}_{32}$ with a predominance in long-chain alcohols ($>n\text{C}_{20}$) in all
253 birches. As short chain-lengths *n*-alcohol homologues ($<n\text{C}_{20}$) are primarily synthesised by
254 microbes or algae [69], we based epicuticular wax analysis on the long-chain homologues. The
255 primary *n*-alcohols showed the closest range in overall concentrations of all lipid classes (Fig.
256 1). The cumulative *n*-alcohol concentration extended from $168.5 \pm 22.0 \mu\text{g/g}$ in *B. pendula* to
257 $436.4 \pm 84.7 \mu\text{g/g}$ in *B. humilis*. The three birches *B. humilis*, *B. pubescens* and *B. pendula*
258 revealed a gradually decreasing concentration with increasing chain-lengths, whereby *B.*
259 *humilis* maximized at $n\text{C}_{20}$ with $209.0 \pm 40.2 \mu\text{g/g}$ d.w. and the latter two at $n\text{C}_{22}$ with
260 153.5 ± 12.9 and $79.2 \pm 10.0 \mu\text{g/g}$ d.w., respectively. *B. nana* was characterized by a narrower
261 distribution, peaking at $n\text{C}_{28}$ with $99.7 \pm 23.3 \mu\text{g/g}$ d.w.. Our results demonstrate that the
262 concentration of *n*-alcohols either increased or decreased gradually with increasing carbon
263 chain-length with only one homologue being dominant (Fig. 1). Both tree birches, *B. pubescens*
264 and *B. pendula*, revealed similar alcohol patterns and differed in absolute concentrations only.

265 In contrast to the tree birches, however, the two shrub birches showed a markedly different *n*-
266 alcohol distribution and were well distinguishable.
267 Diefendorf et al. (2011, 2015) reported that the average concentrations of *n*-alcohols in leaves
268 of deciduous angiosperm trees from the U.S. commonly were twice as high as those of *n*-
269 alkanes. In our study *B. nana* exclusively revealed about 10 times higher concentrations of *n*-
270 alcohols compared to *n*-alkanes. The other three species produced two to four times lower
271 amounts of *n*-alcohols than *n*-alkanes. Previous studies have shown that grasses are mainly
272 characterized by *n*C₂₆, *n*C₂₈ and *n*C₃₂ alcohols [67,70]. This corresponds closely to generally
273 low amounts of these homologues in favour of the high proportions of the shorter alcohols *n*C₂₀
274 or *n*C₂₂ in *B. humilis*, *B. pubescens* and *B. pendula*. Only the leaves of *B. nana* produced a
275 higher proportion of *n*C₂₆ and *n*C₂₈ alcohols.

276

277 ***n*-alkanoic acids of *Betula* epicuticular wax**

278 The *n*-alkanoic acid abundances were characterized by a strong even-over-odd dominance in
279 carbon chain-lengths in the range of *n*C₁₂ to *n*C₃₀. Similar to the *n*-alcohols, short-chain
280 homologues (<*n*C₂₀) were not significant for higher plants since these compounds are also
281 produced by a variety of organisms like bacteria and algae, or derive from cellular membranes
282 rather than waxes. Here, in all four birches species alkanolic acids in the range of *n*C₁₂ to *n*C₃₀
283 were observed to peak at *n*C₁₆ or *n*C₂₈. In the range of the long-chain fatty acids (>*n*C₂₀), the
284 four *Betula* species maximized exclusively at *n*C₂₈, whereby their concentrations differed by
285 two orders of magnitude (Fig. 1). Thus, *B. nana* yielded only 1.75±2.2 µg/g d.w. of *n*C₂₈, while
286 *B. humilis* produced 201.2±13.4 µg/g d.w. of C₂₈. *B. pubescens* and *B. pendula* showed
287 intermediate concentrations of 34.3±2.4 and 34.7±4.0 µg/g d.w., respectively. *B. nana*
288 produced more short chain alkanolic acids than long-chain homologues, with highest
289 concentration at *n*C₁₆ with 44.3±6.1 µg/g d.w. and *n*C₁₈ with 20.8±1.3 µg/g d.w..

290 The relative distribution patterns of long-chain alkanolic acids in the four birches were too
291 similar to allow for differentiation. These basic findings were consistent with a litter and topsoil
292 transect experiment in which deciduous forest sites also showed a dominance of nC_{28} alkanolic
293 acids and differed from conifer (nC_{24}) and grasslands sites (nC_{32} and nC_{34}) [71]. Therefore, C_{28}
294 alkanolic acid preponderance may serve to distinguish wax lipid inputs of birches from those
295 of grasses and conifers, like pines. This may be applicable for sediments in periods such as the
296 Late Glacial and Early Holocene in Central Europe, where successions were characterised by
297 a minor diversity in plant species. In contrast, Diefendorf et al. (2011) observed that the n -
298 alkanolic acid distributions across plant groups from the east coast of the USA, both
299 angiosperms and gymnosperms, were similar with no dominant homologue present.

300

301 ***n*-alkyl esters of *Betula* epicuticular wax**

302 Wax esters are dimeric wax compounds build by a n -alcohol and a n -alkanoic acid moiety,
303 whereby each n -alkyl ester isomer can be composed of several different combinations of n -
304 alcohol and n -alkanoic acid homologues (S2) [72]. Saturated wax ester homologues were
305 identified according to their characteristic molecular ions (M^+) [73]. Straight-chain wax esters
306 in the range of nC_{38} to nC_{48} were detected in all four species, which is typical for higher plants
307 [74] (Fig. 1). Additionally, *B. humilis* produced minor quantities of the nC_{36} homologue. The
308 alkyl ester composition of *B. nana* maximized at nC_{44} with $104.2 \pm 33.4 \mu\text{g/g d.w.}$ with an almost
309 normal distribution. *B. humilis* peaked at nC_{36} with $1047.1 \pm 254.0 \mu\text{g/g d.w.}$, followed by a
310 linear decrease in concentration of longer-chain wax esters up to nC_{48} . Wax esters chain lengths
311 of *B. pubescens* and *B. pendula* leaves ranged from nC_{38} to nC_{46} , whereby the concentrations
312 decreased with an increase in chain-lengths. Concentrations of nC_{38} wax ester of *B. pubescens*
313 and *B. pendula* yielded 535.7 ± 99.4 and $122.7 \pm 35.5 \mu\text{g/g d.w.}$, respectively.

314

315 **Isomer distribution of wax esters and input to free *n*-alkanoic acids** 316 **and *n*-alcohol amount**

317 The mass spectral analysis of wax esters by GC/MS allowed to investigate their corresponding
318 bound *n*-alcohol and *n*-alkanoic acids (Fig. 6, S3-S6 Tables).

319 In all four species, only even-chain alkanolic acids in the range of nC_{14} to nC_{28} and alcohol
320 moieties ranging from nC_{18} to nC_{32} were observed resulting in even-chain alkyl esters. In *B.*
321 *nana*, nC_{20} was the overall dominant ester-bound alkanolic acid moiety with 62.2 $\mu\text{g/g}$ d.w. and
322 nC_{24} the most prominent ester-bound alcohol with 58.9 $\mu\text{g/g}$ d.w.. In the shorter nC_{38} and nC_{40}
323 esters shorter ester-bound alkanolic acids with 14 and 16 carbon atoms as well as shorter ester-
324 bound alcohols like nC_{22} occurred. The ester homologues of *B. humilis*, *B. pubescens* and *B.*
325 *pendula* were dominated by short-chain nC_{16} bound alkanolic acid moieties (841.5, 368.8, 75.7
326 $\mu\text{g/g}$ d.w.), while nC_{20} was the most dominant alkanolic acid homologue in the long-chain
327 alkanolic acid fraction (190.6, 38.0, 33.4 $\mu\text{g/g}$ d.w.). However, compared to the short-chain
328 alkanolic acids, the long-chain homologues were less abundant in concentrations up to factor of
329 10. The major esterified alcohol within the three species varied significantly. *B. humilis* was
330 dominated by nC_{20} (744.2 $\mu\text{g/g}$ d.w.), *B. pubescens* by nC_{22} (346.6 $\mu\text{g/g}$ d.w.) and *B. pendula*
331 by nC_{24} (82.7 $\mu\text{g/g}$ d.w.) and nC_{22} (81.2 $\mu\text{g/g}$ d.w.) bound alcohols.

332 The bound *n*-alkanoic acid and *n*-alcohol moieties of wax esters might be released during
333 hydrolysis upon incorporation of alkyl esters into soil or during early burial stages in sediments.
334 As consequence, the amount of hydrolysis-released, previously ester-bound *n*-alkanoic acids
335 and *n*-alcohols in a sediment will impact on the quantity and distribution pattern of free *n*-
336 alkanolic acids and *n*-alcohols derived from leaf waxes [70] and needs to be considered in
337 paleovegetation reconstruction. However, intact wax esters can survive in sediments and can
338 be used for paleovegetation reconstruction [63,75,76] as well.

339 To test for the potential release of alcohols and acids from esters two different scenarios were
340 calculated. In the first scenario, 50% of the esterified *n*-alkanoic acids and *n*-alcohols were
341 released and added to their corresponding free homologues (Fig. 2). In the second scenario, the
342 maximum release of 100% of the bound lipids and addition to the free analogues was used.
343 Since the wax esters of all four birches consisted mainly of short-chain fatty acids, they do not
344 significantly increase the pool of long-chain fatty acids ($>nC_{20}$) typical for terrestrial higher
345 plants. Solely in *B. nana*, the alkanolic acid distribution changed from a previously rather
346 balanced distribution of nC_{20} to nC_{28} with 1 to 2 $\mu\text{g/g}$ d.w. to a dominance of nC_{20} with >60
347 $\mu\text{g/g}$ d.w., due to the release of the esterified homologues. A dominance of nC_{28} alkanolic acid
348 was still observed for the other three birches when 50% of the esterified alkanolic acids had
349 been released. Only upon 100% release of the bound long-chain fatty acids, a bimodal
350 distribution maximizing at nC_{20} and nC_{28} could be noted for *B. pubescens*, which would
351 complicate a source identification in sediments.

352 The bound *n*-alcohols of the alkyl esters in the four European birches ranged from nC_{20} to nC_{32} .
353 Sometimes, the dominant esterified *n*-alcohol was found to be the same as the dominant free
354 homologue in the same species (Fig. 3). For example, in *B. humilis* nC_{20} and in *B. pubescens*
355 nC_{22} were the dominant free and esterified *n*-alcohol, respectively. The release of 100 % bound
356 *n*-alcohols of both species increased the total amount by about 25%. However, the relative
357 distributions remained comparable. The previously identified dominance of the free nC_{22}
358 alcohol in *B. pendula* was reduced by the release of a high proportion of nC_{24} . In contrast, the
359 distribution in *B. nana* changed from a dominance of nC_{28} to a bimodal distribution maximizing
360 at nC_{24} and nC_{28} due to the addition of ester-bound *n*-alcohols. The dominance of free nC_{22}
361 alcohol in *B. pendula* was reduced by the release of the high proportion of nC_{24} .

362 Our model indicates that the decay of the *n*-alkyl ester can significantly affect the original free
363 lipid composition of birch leaves, which in sediments may complicate an unambiguous
364 assignment based on *n*-alcohol and *n*-alkanoic acids.

365

366 **Fig. 2: Distribution of esterified (bound) alkanolic acids in the alkyl esters and their**
367 **corresponding free homologues in the same leaf.** The two lower rows depict the summed
368 concentration of free fatty acids plus an additional 50% or 100 % bound fatty acids released by
369 hydrolysis, respectively.

370

371

372 **Fig. 3: Distribution of esterified (bound) alcohols in the alkyl esters and their**
373 **corresponding free homologues in the same leaf.** The two lower rows depict the summed
374 concentration of free fatty acids plus an additional 50% or 100 % bound fatty acids released by
375 hydrolysis, respectively.

376

377

378 **Wax lipids from *Betula* grown in Kiel compared with literature** 379 **data**

380 To consolidate the application of the lipid composition of the four European birches for
381 paleovegetation reconstruction and chemotaxonomic differentiation, we compared our wax
382 lipid data of birch trees grown in the Botanical Garden in Kiel with previously published data
383 (Table 1). However, solely the *n*-alkane composition of *B. nana*, *B. pubescens* and *B. pendula*
384 can be compared, as to the best of our knowledge, the other wax lipid classes were not
385 examined in full in previous studies. To our best knowledge, there are no previous studies on
386 the wax lipid composition of *B. humilis* leaves, thus we do not have any complementary data

387 for comparison. For a better comparison of published data, we have recalculated the
388 distributions given in absolute concentrations into relative abundances, to exclude variation in
389 absolute concentration due to different extraction techniques or analytical protocols. Different
390 extraction techniques and analytic procedures used in the cited studies are briefly described
391 here:

- 392 - Extraction of epicuticular waxes by immersion of the whole leaf into solvent mixture with or
393 without ultrasonic bath, e.g. DCM:hexane (1:1) or pure DCM (our study, [77]) ;
- 394 - Prior to extraction, grinding or milling of leaves into a fine powder, followed by ASE
395 (extraction under elevated temperature and pressure) or Soxhlet extraction [78–80]; here,
396 intra-cuticular waxes potentially have been extracted;
- 397 - Hydrolysis (10% KOH in ethanol) of ground leaves to extract bound lipids [81]; esterified
398 alkanolic acids and alcohols were released and increased the pool of their free homologues.

399 Most leaves from *B. nana* are characterized by a variable distribution of *n*-alkanes ranging
400 from *n*C₂₃ to *n*C₃₃. Samples from very northern latitudes such as Greenland, Alaska, Siberia
401 and Norway had a high proportion of *n*C₂₇ to *n*C₃₁ homologues [80–82]. Conversely, *B. nana*
402 leaves from Siberia and Norway can be distinguished from the other *B. nana* samples as these
403 showed significant abundances of mid-chain *n*C₂₃ and *n*C₂₅ alkanes depressing relative
404 amounts of C₂₉ and C₃₁ homologues [83,84]. Leaf *n*-alkanes of *B. pubescens* from Scotland
405 were bimodally distributed with maxima at *n*C₂₇ and *n*C₃₁ [85]. In contrast, a unimodal
406 distribution with a maximum at *n*C₂₇ was reported from Pagani et al. (2006), Ronkainen et al.
407 (2015), and Balascio et al. (2018), with variable proportions of *n*C₂₃ and *n*C₂₅, respectively. A
408 dominance of C₂₇ alkane in leaves from *B. pubescens* has also been reported from Schwark et
409 al. (2002) and Sachse et al. (2006). The latter author stated that only in birches (both *B.*
410 *pubescens* and *B. pendula*) from northern Scandinavia *n*C₂₇ is the dominant homologue, while
411 species from southern Scandinavia and Germany maximized at *n*C₃₁. This shift in *n*-alkane

412 carbon chain-length was also expressed in an increasing ACL from North to South [87]. In
413 contrast to this, Mayes et al. (1994) found a prevalence of C₂₅ in leaves from Norway.
414 Similar to the leaves of *B. pubescens*, those of *B. pendula* contained high proportions of C₂₅, if
415 the distribution maximized at nC₂₇ and then constituted only minor long-chain homologues
416 with more than 29 carbon atoms [78,79,88,89]. Two *B. pendula* species from Estonia and the
417 UK, when grown under artificial laboratory conditions had a distinct bimodal distribution with
418 maxima at C₂₇ and C₃₁ [77,90].
419 Due to the complexity of the *n*-alkane patterns both within a species and between different
420 species, we subdivided the data according to distributions of *n*-alkanes in each species into
421 three groups to improve comparison. The published data of each species were subdivided into
422 two groups (type I and type II) of similar composition and compared with the lipid distribution
423 of the birches from Kiel (Fig. 4, Table 2). Type I of each species had a wax lipid composition
424 similar to the *Betula* species from Kiel and was characterized by a dominance of long-chain *n*-
425 alkanes (nC₂₇, nC₂₉, nC₃₁). In contrast, type II of each species was defined by a high presence
426 of mid-chain *n*-alkanes (nC₂₃, nC₂₅), but also nC₂₇, and only minor quantities of long-chain *n*-
427 alkanes with more than 29 carbon atoms.
428 All *Betula* tree species from Kiel and from globally distributed type I were distinct from grasses
429 and shrubs by a prominent prevalence of the nC₂₇ alkane (Fig. 1 and 4). Grasses are mainly
430 dominated by very long-chain *n*-alkanes with nC₃₁ and nC₃₃ or even nC₃₅, and therefore are
431 characterized by a high ACL (>30) [44,49,67]. Typical pioneer shrubs of the late glacial period
432 such as *Artemisia sp.* or *Junipers sp.* are also characterized by a dominance at nC₃₁ to nC₃₅,
433 which is not prevalent in *Betula* species [45,91,92]. Other prominent species such as *Pinus sp.*,
434 which are probably the most widely spread conifer species in Europe, synthesize *n*-alkanes in
435 the range from nC₂₇ and nC₃₁ [45,93]. However, the quantitative amounts are significantly

436 lower, pointing to subordinate proportions of sedimentary *n*-alkanes originating from pines
437 [47].

438 The type II birch species had *n*-alkane distributions similar to aquatic macrophytes and non-
439 emergent (submerged and floating) aquatic plants, with a prevalence of mid-chain *n*-alkanes
440 (nC_{23} , nC_{25}). These homologues can be a major source to geological archives, especially in
441 lake sediments, often expressed in the P_{aq} proxy [54,94]. It has been postulated that terrestrial
442 plants correspond to $P_{aq} < 0.1$, emergent macrophytes to $P_{aq} 0.1 - 0.4$ and non-emergent
443 macrophytes to $P_{aq} 0.4 - 1.0$ to [54]. Each type II birch species, as well as *B. humilis* from our
444 study, had a P_{aq} value above 0.75 corresponding to a non-emergent macrophyte. Therefore,
445 when applying the P_{aq} proxy in the study of lake sediments receiving birch input, it must be
446 considered that mid-chain *n*-alkanes may derive from either aquatic macrophytes or
447 alternatively from birch trees.

448 *n*-Alkane abundance or chain-length based indicators like ACL have been used as a proxy for
449 temperature, aridity, geographic location, or vapour pressure deficit [95–98]. The variation of
450 the ACL compiled for *B. nana* did not show a trend with climatic drivers. The *B. nana* trees
451 listed, mostly derived from cold environments such as Alaska, Greenland or Siberia and varied
452 in their ACL between 26.4 and 28.9 [80,82,83]. Even two samples both originating from
453 Norway varied by over 2 units in their ACL [81,84]. The *B. nana* investigated from Kiel, and
454 therefore from the warmest region, did not reveal the highest ACL, but rather values between
455 those of leaves from Greenland and Alaska. The ACL distributions of *B. pubescens* revealed
456 higher values in samples from a moderate climate (Kiel, Scotland), whereas samples from
457 colder regions (Siberia, Norway) had a lower ACL. Under certain assumptions, this can be
458 attributed a geographical or temperature effect. The origin for the *B. pubescens* leaves from the
459 study by Pagani et al. (2006) were not reported. Similar to *B. nana*, no latitudinal or temperature
460 trend in the *n*-alkane distribution of *B. pendula* wax was observed.

461 Overall, a high variability of wax *n*-alkanes within the individual species was noted without a
462 temperature or geographical trend. This may suggest that genetic differences between the
463 populations control wax lipid composition, preferentially. It is conceivable that not only "pure-
464 bred" birches of the respective species were examined in these studies, but also subspecies or
465 varieties. For example, *Betula pubescens* has several varieties that occur naturally in a narrow
466 space like *var. pubescens*, *var. fragrans*, *var. litwinowii* and *var. pumila* [15]. Wild
467 hybridization may also affect leaf wax composition, whereby hybridization readily occurs
468 between species with the same chromosome number like *B. pendula* x *B. nana* (diploid x
469 diploid), but there are also reports of interploidy-level hybrids like *pendula* x *B. pubescens*
470 (diploid x tetraploid) [99,100]. Therefore, future studies may address the association between
471 ploidy level and wax lipid composition of *Betula* species to investigate species determination.

472

473 **Figure 4: Relative abundances of *n*-alkane patterns for *Betula nana*, *Betula humilis*, *Betula***
474 ***pubescens* and *Betula pendula*.** The samples from our study (Kiel) were compared with literature data
475 previously divided into two subgroups (Type I and Type II; see text for details) with similar
476 compositions. Note, to the best of our knowledge there are no previous studies reporting the epicuticular
477 wax composition of *Betula humilis*.

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485 **Table 1: Relative *n*-alkane abundances of all *Betula* species**

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487

Species	Article	Location	Typ	Relative abundance %																	ACL
				23	24	25	26	27	28	29	30	31	32	33	34	35					
<i>B. nana</i>	Weber & Schwark (2019)	Kiel	Kiel	0	0	9	3	37	3	14	2	32	0	0	0	0	0	28.49			
<i>B. nana</i>	Berke et al. (2019)	Greenland	I	9	7	14	10	17	1	15	1	24	1	2	0	0	0	27.95			
<i>B. nana</i>	Daniels et al. (2017)	Alaska (USA)	I	1	4	5	9	24	3	17	3	26	3	5	0	0	0	28.94			
<i>B. nana</i>	Mayes et al. (1994)	Norway	I	12	4	10	7	20	0	19	1	23	0	2	0	0	0	27.88			
<i>B. nana</i>	Ronkainen et al. (2015)	Siberia (Russia)	II	20	1	22	1	34	1	7	1	12	0	1	0	0	0	26.45			
<i>B. nana</i>	Balascio et al. (2018)	Norway	II	30	0	27	0	30	0	5	0	6	0	1	0	0	0	25.65			
<i>B. humilis</i>	Weber & Schwark (2019)	Kiel	Kiel	0	0	23	0	72	0	5	0	0	0	0	0	0	0	26.65			
<i>B. pubescens</i>	Weber & Schwark (2019)	Kiel	Kiel	0	0	11	1	15	0	11	1	49	1	12	0	0	0	29.72			
<i>B. pubescens</i>	Rao et al. (2003)	Scotland (UK)	I	1	1	16	3	25	1	16	0	31	2	4	0	0	0	28.53			
<i>B. pubescens</i>	Ronkainen et al. (2015)	Siberia (Russia)	II	19	1	25	2	42	1	5	0	4	0	0	0	0	0	26.00			
<i>B. pubescens</i>	Pagani et al. (2006)	?	II	5	0	23	0	73										26.36			
<i>B. pubescens</i>	Mayes et al. (1994)	Norway	II	35	0	48	0	4	0	9	0	3	0	0	0	0	0	24.94			
<i>B. pubescens</i>	Balascio et al. (2018)	Norway	II	25	0	27	0	43	0	3	0	1	0	0	0	0	0	25.53			
<i>B. pendula</i>	Weber & Schwark (2019)	Kiel	Kiel	2	0	24	1	29	0	7	0	32	0	3	0	0	0	28.07			
<i>B. pendula</i>	Huang et al. (1999)	Solar Dome (UK)	I	0	0	20	0	25	0	12	0	37	0	7	0	0	0	28.72			
<i>B. pendula</i>	Lihavainen et al. (2017)	Estonia	I	2	1	12	2	18	4	7	4	19	6	14	3	8	0	29.60			
<i>B. pendula</i>	Zech et al. (2010)	Russia	II	0	0	30	2	50	1	5	1	10	1	1	0	0	0	26.96			
<i>B. pendula</i>	Dawson et al. (2004)	UK	II	6	0	34	0	38	0	6	0	12	0	3	0	0	0	26.88			
<i>B. pendula</i>	Tarasov et al. (2013)	Siberia (Russia)	II	0	0	45	3	49	1	2	0	0	0	0	0	0	0	26.09			
<i>B. pendula</i>	van den Bos et al. (2018)	Netherlands	II	11	0	31	0	40	0	6	0	11	0	1	0	0	0	26.56			

Table 2:

Species	Typ	Relative abundance (%)													ACL
		23	24	25	26	27	28	29	30	31	32	33	34	35	
<i>B. nana</i>	Kiel	0	0	9	3	37	3	14	2	32	0	0	0	0	28.49
<i>B. nana</i>	I	7	5	10	9	20	1	17	2	24	1	3	0	0	28.24
<i>B. nana</i>	II	25	1	25	1	32	1	6	0	9	0	1	0	0	26.04
<i>B. humilis</i>	Kiel	0	0	23	0	72	0	5	0	0	0	0	0	0	26.65
<i>B. pubescens</i>	Kiel	0	0	11	1	15	0	11	1	49	1	12	0	0	29.72
<i>B. pubescens</i>	I	1	1	16	3	25	1	16	0	31	2	4	0	0	28.53
<i>B. pubescens</i>	II	21	0	31	0	40	0	6	0	3	0	0	0	0	25.79
<i>B. pendula</i>	Kiel	2	0	24	1	29	0	7	0	32	0	3	0	0	28.07
<i>B. pendula</i>	I	1	1	16	1	21	2	10	2	28	3	10	2	4	29.11
<i>B. pendula</i>	II	4	0	35	1	44	1	5	0	8	0	1	0	0	26.62

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494 **Analytical and extraction protocols used in previous *Betula* studies**

495 Different analytic protocols have been used to extract the plant lipids as briefly described
 496 above. Previous studies have shown that the length of the extraction time, as well as the solvent
 497 used, had an influence on extraction yield or lipid extract composition [37,46]. Thus, *n*-alkanes
 498 and *n*-alcohols were extracted earlier than *n*-alkanoic acids and long-chain homologues earlier
 499 than the shorter ones [101]. Jetter et al. (2008) indicated extraction yields of *n*-alkanes
 500 depended on polarity of binary solvent mixtures. Moreover, saponification upon extraction
 501 [81], hydrolyzed wax esters leading to enhanced release of bound *n*-alcohols and *n*-alkanoic
 502 acids adding to the proportion of the free homologues. Since this comparative investigation
 503 used *n*-alkane distributions from different studies with different extraction methods, the results
 504 are not unequivocally comparable. For a better comparability of future work, the influence of
 505 the extraction method on the other lipid classes including *n*-alcohols, *n*-alkanoic acids and *n*-
 506 alkyl esters should be investigated and a standard extraction protocol established.

507

508 Conclusion

509 The leaves of four *Betula* species, *B. nana*, *B. humilis*, *B. pubescens*, *B. pendula*, which are
510 endemic in Europe were studied, aiming to investigate their epicuticular wax lipid composition.

511 The following conclusions can be drawn from this study. The *n*-alkane compositions in leaves
512 of *Betula* species from Kiel were found to be specific, allowing unambiguous differentiation.

513 *Betula* wax *n*-alcohol and *n*-alkyl ester composition allowed a distinction to be made between
514 *B. nana*, *B. humilis* and the two birch trees, however the latter two cannot be easily
515 distinguished from each other due to a similar fingerprint. The *n*-alkanoic acids seemed to be
516 less suitable for species differentiation since all four species were dominated by the C₂₈
517 alkanolic acid, however with variations in concentration of about two orders of magnitude. A
518 flowchart (Fig. 5) provides a simple means for discrimination of epicuticular waxes from the
519 four birches from Kiel University.

520 The *n*-alkyl esters consisted of different isomers with varying *n*-alcohol and *n*-alkanoic acid
521 moieties. In the species *B. humilis* and *B. pubescens*, the dominant esterified alcohol also was
522 the dominant free alcohol, therefore the *n*-alcohol patterns in sediments would not be disturbed
523 by hydrolysis of the wax esters. In *B. nana* and *B. pendula* the *n*-alcohol distribution changed
524 substantially upon ester hydrolysis, when bound homologues were released. Due to the
525 preponderance of short-chain ester-bound alkanolic acids in wax esters, the distribution of free
526 long-chain alkanolic acids was only slightly impaired. The ratio was influenced in *B. nana* only,
527 as large amounts of bound C₂₀ were released.

528 When comparing the *n*-alkane composition of the *Betula* waxes collected in Kiel with
529 published data, no trend in geographical location or temperature could be identified. It appears
530 that *Betula* wax composition is genetically controlled, and differences occur due to presence of
531 plant hybrids or variants.

532

533 **Figure 5: Decision making tree for differentiation of four European *Betula* species based**
534 **on epicuticular wax composition**

535

536

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552 **References**

- 553 1. Crane PR, Stockey RA. *Betula* leaves and reproductive structures from the Middle Eocene of
554 British Columbia, Canada. *Can J Bot*. 1987;65: 2490–2500. doi:10.1139/b87-338
- 555 2. Järvinen P, Palme A, Orlando Morales L, Lannenpaa M, Keinanen M, Sopanen T, et al.
556 Phylogenetic relationships of *Betula* species (Betulaceae) based on nuclear ADH and
557 chloroplast matK sequences. *Am J Bot*. 2004;91: 1834–1845. doi:10.3732/ajb.91.11.1834
- 558 3. Schenk MF, Thienpont C-N, Koopman WJM, Gilissen LJWJ, Smulders MJM. Phylogenetic
559 relationships in *Betula* (Betulaceae) based on AFLP markers. *Tree Genet Genomes*. 2008;4:
560 911. doi:10.1007/s11295-008-0162-0
- 561 4. Bina H, Yousefzadeh H, Ali SS, Esmailpour M. Phylogenetic relationships, molecular
562 taxonomy, biogeography of *Betula*, with emphasis on phylogenetic position of Iranian
563 populations. *Tree Genet Genomes*. 2016;12: 84. doi:10.1007/s11295-016-1037-4
- 564 5. Julkunen-Tiitto R, Rousi M, Bryant J, Sorsa S, Keinänen M, Sikanen H. Chemical diversity of
565 several Betulaceae species: comparison of phenolics and terpenoids in northern birch stems.
566 *Trees*. 1996;11: 16. doi:10.1007/s004680050053
- 567 6. Keinänen M, Julkunen-Tiitto R, Rousi M, Tahvanainen J. Taxonomic implications of phenolic
568 variation in leaves of birch (*Betula* L.) species. *Biochem Syst Ecol*. 1999;27: 243–254.
569 doi:10.1016/S0305-1978(98)00086-6
- 570 7. Orav A, Arak E, Boikova T, Raal A. Essential oil in *Betula* spp. leaves naturally growing in
571 Estonia. *Biochem Syst Ecol*. 2011;39: 744–748. doi:10.1016/j.bse.2011.06.013
- 572 8. Depciuch J, Kasprzyk I, Drzymała E, Parlinska-Wojtan M. Identification of birch pollen
573 species using FTIR spectroscopy. *Aerobiologia (Bologna)*. 2018;34: 525–538.
574 doi:10.1007/s10453-018-9528-4
- 575 9. Furlow JJ. The genera of Betulaceae in the southeastern United States. *J Arnold Arboretum*.
576 1990;71: 1–67. doi:10.5962/bhl.part.24925
- 577 10. Maliouchenko O, Palmé AE, Buonamici A, Vendramin GG, Lascoux M. Comparative
578 phylogeography and population structure of European *Betula* species, with particular focus on

- 579 B. pendula and B. pubescens. J Biogeogr. 2007;34: 1601–1610. doi:10.1111/j.1365-
580 2699.2007.01729.x
- 581 11. Wang N, McAllister HA, Bartlett PR, Buggs RJA. Molecular phylogeny and genome size
582 evolution of the genus *Betula* (Betulaceae). Ann Bot. 2016;117: 1023–1035.
583 doi:10.1093/aob/mcw048
- 584 12. Winkler H. Betulaceae Das Pflanzenreich. 39th ed. Engler A, editor. 1904.
- 585 13. De Jong PC. An introduction to *Betula*: Its morphology, evolution, classification and
586 distribution with a survey of recent work. IDS *Betula* Symp Int Dendrol Soc Susses, UK.
587 1993.
- 588 14. Skvortsov AK. A new system of the genus *Betula* L. Bull Mosc Natur Soc. 2002;107: 73–76.
- 589 15. Ashburner K, McAllister HA. The Genus *Betula*: A Taxonomic Revision of Birches. 2013.
- 590 16. Freund H, Birks HH, Birks HJB. The identification of wingless *Betula* fruits in Weichselian
591 sediments in the Gross Todtshorn borehole (Lower Saxony, Germany) – the occurrence of
592 *Betula humilis* Schrank. Veg Hist Archaeobot. 2001;10: 107–115. doi:10.1007/PL00006919
- 593 17. van Dinter M, Birks HH. Distinguishing fossil *Betula nana* and *B. pubescens* using their
594 wingless fruits: implications for the late-glacial vegetational history of western Norway. Veg
595 Hist Archaeobot. 1996;5: 229–240. doi:10.1007/BF00217500
- 596 18. Tsuda Y, Semerikov V, Sebastiani F, Vendramin GG, Lascoux M. Multispecies genetic
597 structure and hybridization in the *Betula* genus across Eurasia. Mol Ecol. 2017;26: 589–605.
598 doi:10.1111/mec.13885
- 599 19. Thomson AM, Dick CW, Pascoini AL, Dayanandan S. Despite introgressive hybridization,
600 North American birches (*Betula* spp.) maintain strong differentiation at nuclear microsatellite
601 loci. Tree Genet Genomes. 2015;11: 101. doi:10.1007/s11295-015-0922-6
- 602 20. Thórsson AT, Salmela E, Anamthawat-Jónsson K. Morphological, cytogenetic, and molecular
603 evidence for introgressive hybridization in birch. J Hered. 2001;92: 404–8.
604 doi:10.1093/jhered/92.5.404
- 605 21. Tralau H. The recent and fossil distribution of some boreal and arctic montane plants in
606 Europe. Ark. Bot. Ser. 2; 1963.

- 607 22. Krüger S, Damrath M. In search of the Bølling-Oscillation: a new high resolution pollen
608 record from the locus classicus Lake Bølling, Denmark. *Veg Hist Archaeobot.* 2019;
609 doi:10.1007/s00334-019-00736-3
- 610 23. Eusterhues K, Lechterbeck J, Schneider J, Wolf-Brozio U. Late- and Post-Glacial evolution of
611 Lake Steisslingen. *Palaeogeogr Palaeoclimatol Palaeoecol.* 2002;187: 341–371.
612 doi:10.1016/S0031-0182(02)00486-8
- 613 24. Lotter AF. Late-glacial and Holocene vegetation history and dynamics as shown by pollen and
614 plant macrofossil analyses in annually laminated sediments from Soppensee, central
615 Switzerland. *Veg Hist Archaeobot.* 1999;8: 165–184. doi:10.1007/BF02342718
- 616 25. Lotter AF, Eicher U, Siegenthaler U, Birks HJB. Late-glacial climatic oscillations as recorded
617 in Swiss lake sediments. *J Quat Sci.* 1992;7: 187–204. doi:10.1002/jqs.3390070302
- 618 26. Veski S, Amon L, Heinsalu A, Reitalu T, Saarse L, Stivrins N, et al. Lateglacial vegetation
619 dynamics in the eastern Baltic region between 14,500 and 11,400calyrBP: A complete record
620 since the Bølling (GI-1e) to the Holocene. *Quat Sci Rev.* 2012;40: 39–53.
621 doi:10.1016/j.quascirev.2012.02.013
- 622 27. Tarasov PE, Bezrukova E V., Krivonogov SK. Late Glacial and Holocene changes in
623 vegetation cover and climate in southern Siberia derived from a 15 kyr long pollen record from
624 Lake Kotokel. *Clim Past.* 2009;5: 285–295. doi:10.5194/cp-5-285-2009
- 625 28. Mortensen MF, Birks HH, Christensen C, Holm J, Noe-Nygaard N, Odgaard BV, et al.
626 Lateglacial vegetation development in Denmark – New evidence based on macrofossils and
627 pollen from Slotseng, a small-scale site in southern Jutland. *Quat Sci Rev.* 2011;30: 2534–
628 2550. doi:10.1016/j.quascirev.2011.04.018
- 629 29. Rösch M, Lechterbeck J. Seven Millennia of human impact as reflected in a high resolution
630 pollen profile from the profundal sediments of Litzelsee, Lake Constance region, Germany.
631 *Veg Hist Archaeobot.* 2016;25: 339–358. doi:10.1007/s00334-015-0552-9
- 632 30. Jahns S. Late-glacial and Holocene woodland dynamics and land-use history of the Lower
633 Oder valley, north-eastern Germany, based on two, AMS14C-dated, pollen profiles. *Veg Hist*
634 *Archaeobot.* 2000;9: 111–123. doi:10.1007/BF01300061

- 635 31. Birks HJB. The Identification of *Betula nana* Pollen. *New Phytol.* 1968;67: 309–314.
636 doi:10.1111/j.1469-8137.1968.tb06386.x
- 637 32. Mäkelä EM. Size distinctions between *Betula* pollen types — A review. *Grana.* 1996;35: 248–
638 256. doi:10.1080/00173139609430011
- 639 33. Caseldine C. Changes in *Betula* in the Holocene record from Iceland—a palaeoclimatic record
640 or evidence for early Holocene hybridisation? *Rev Palaeobot Palynol.* 2001;117: 139–152.
641 doi:10.1016/S0034-6667(01)00082-3
- 642 34. Karlsdóttir L, Thórsson ÆT, Hallsdóttir M, Sigurgeirsson A, Eysteinnsson T, Anamthawat-
643 Jónsson K. Differentiating pollen of *Betula* species from Iceland. *Grana.* 2007;46: 78–84.
644 doi:10.1080/00173130701237832
- 645 35. de Klerk P, Theuerkauf M, Joosten H. Vegetation, recent pollen deposition, and distribution of
646 some non-pollen palynomorphs in a degrading ice-wedge polygon mire complex near
647 Pokhodsk (NE Siberia), including size-frequency analyses of pollen attributable to *Betula*. *Rev*
648 *Palaeobot Palynol.* 2017;238: 122–143. doi:10.1016/j.revpalbo.2016.11.015
- 649 36. Jenks MA, Joly RJ, Peters PJ, Rich PJ, Axtell JD, Ashworth EN. Chemically Induced Cuticle
650 Mutation Affecting Epidermal Conductance to Water Vapor and Disease Susceptibility in
651 *Sorghum bicolor* (L.) Moench. *Plant Physiol.* 1994;105: 1239–1245.
652 doi:10.1104/pp.105.4.1239
- 653 37. Jetter R, Kunst L. Plant surface lipid biosynthetic pathways and their utility for metabolic
654 engineering of waxes and hydrocarbon biofuels. *Plant J.* 2008;54: 670–683.
655 doi:10.1111/j.1365-313X.2008.03467.x
- 656 38. Sieber P, Schorderet M, Ryser U, Buchala A, Kolattukudy P, Métraux J-P, et al. Transgenic
657 *Arabidopsis* Plants Expressing a Fungal Cutinase Show Alterations in the Structure and
658 Properties of the Cuticle and Postgenital Organ Fusions. *Plant Cell.* 2000;12: 721–737.
659 doi:10.1105/tpc.12.5.721
- 660 39. Long LM, Patel HP, Cory WC, Stapleton AE. The maize epicuticular wax layer provides UV
661 protection. *Funct Plant Biol.* 2003;30: 75. doi:10.1071/FP02159
- 662 40. Eglinton G, Hamilton RJ. Leaf Epicuticular Waxes. *Science* (80-). 1967;156: 1322–1335.

- 663 doi:10.1126/science.156.3780.1322
- 664 41. Eglinton G, Gonzalez AG, Hamilton RJ, Raphael RA. Hydrocarbon constituents of the wax
665 coatings of plant leaves: A taxonomic survey. *Phytochemistry*. 1962;1: 89–102.
666 doi:10.1016/S0031-9422(00)88006-1
- 667 42. Herbin GA, Robins PA. Patterns of variation and development in leaf wax alkanes.
668 *Phytochemistry*. 1969;8: 1985–1998. doi:10.1016/S0031-9422(00)88085-1
- 669 43. Gülz P-G. Epicuticular Leaf Waxes in the Evolution of the Plant Kingdom. *J Plant Physiol*.
670 1994;143: 453–464. doi:10.1016/S0176-1617(11)81807-9
- 671 44. Maffei M. Chemotaxonomic significance of leaf wax alkanes in the gramineae. *Biochem Syst*
672 *Ecol*. 1996;24: 53–64. doi:10.1016/0305-1978(95)00102-6
- 673 45. Schwark L, Zink K, Lechterbeck J. Reconstruction of postglacial to early Holocene vegetation
674 history in terrestrial Central Europe via cuticular lipid biomarkers and pollen records from lake
675 sediments. *Geology*. 2002;30: 463. doi:10.1130/0091-
676 7613(2002)030<0463:ROPTEH>2.0.CO;2
- 677 46. Buschhaus C, Herz H, Jetter R. Chemical Composition of the Epicuticular and Intracuticular
678 Wax Layers on Adaxial Sides of *Rosa canina* Leaves. *Ann Bot*. 2007;100: 1557–1564.
679 doi:10.1093/aob/mcm255
- 680 47. Diefendorf AF, Freeman KH, Wing SL, Graham H V. Production of n-alkyl lipids in living
681 plants and implications for the geologic past. *Geochim Cosmochim Acta*. 2011;75: 7472–
682 7485. doi:10.1016/j.gca.2011.09.028
- 683 48. Diefendorf AF, Leslie AB, Wing SL. Leaf wax composition and carbon isotopes vary among
684 major conifer groups. *Geochim Cosmochim Acta*. 2015;170: 145–156.
685 doi:10.1016/j.gca.2015.08.018
- 686 49. Bush RT, McInerney FA. Leaf wax n-alkane distributions in and across modern plants:
687 Implications for paleoecology and chemotaxonomy. *Geochim Cosmochim Acta*. 2013;117:
688 161–179. doi:10.1016/j.gca.2013.04.016
- 689 50. Mueller-Niggemann C, Schwark L. Chemotaxonomy and diagenesis of aliphatic hydrocarbons
690 in rice plants and soils from land reclamation areas in the Zhejiang Province, China. *Org*

- 691 Geochem. 2015;83–84: 215–226. doi:10.1016/j.orggeochem.2015.03.016
- 692 51. Guo Y, Li JJ, Busta L, Jetter R. Coverage and composition of cuticular waxes on the fronds of
693 the temperate ferns *Pteridium aquilinum*, *Cryptogramma crispa*, *Polypodium glycyrrhiza*,
694 *Polystichum munitum* and *Gymnocarpium dryopteris*. *Ann Bot.* 2018;122: 555–568.
695 doi:10.1093/aob/mcy078
- 696 52. Blumer M, Guillard RRL, Chase T. Hydrocarbons of marine phytoplankton. *Mar Biol.* 1971;8:
697 183–189. doi:10.1007/BF00355214
- 698 53. Cranwell PA, Eglinton G, Robinson N. Lipids of aquatic organisms as potential contributors to
699 lacustrine sediments-II. *Org Geochem.* 1987;11: 513–527. doi:10.1016/0146-6380(87)90007-6
- 700 54. Ficken K., Li B, Swain D., Eglinton G. An n-alkane proxy for the sedimentary input of
701 submerged/floating freshwater aquatic macrophytes. *Org Geochem.* 2000;31: 745–749.
702 doi:10.1016/S0146-6380(00)00081-4
- 703 55. Pancost RD, Baas M, van Geel B, Sinninghe Damsté JS. Biomarkers as proxies for plant
704 inputs to peats: an example from a sub-boreal ombrotrophic bog. *Org Geochem.* 2002;33:
705 675–690. doi:10.1016/S0146-6380(02)00048-7
- 706 56. Shepherd T, Robertson GW, Griffiths DW, Birch ANE. Epicuticular wax ester and
707 triacylglycerol composition in relation to aphid infestation and resistance in red raspberry
708 (*Rubus idaeus* L.). *Phytochemistry.* 1999;52: 1255–1267. doi:10.1016/S0031-9422(99)00414-
709 8
- 710 57. Schouten S, Woltering M, Rijpstra WIC, Sluijs A, Brinkhuis H, Sinninghe Damsté JS. The
711 Paleocene-Eocene carbon isotope excursion in higher plant organic matter: Differential
712 fractionation of angiosperms and conifers in the Arctic. *Earth Planet Sci Lett.* 2007;258: 581–
713 592. doi:10.1016/j.epsl.2007.04.024
- 714 58. Smith F, Wing S, Freeman K. Magnitude of the carbon isotope excursion at the Paleocene–
715 Eocene thermal maximum: The role of plant community change. *Earth Planet Sci Lett.*
716 2007;262: 50–65. doi:10.1016/j.epsl.2007.07.021
- 717 59. Schellekens J, Buurman P. Geoderma n -Alkane distributions as palaeoclimatic proxies in
718 ombrotrophic peat : The role of decomposition and dominant vegetation. *Geoderma.* 2011;164:

- 719 112–121. doi:10.1016/j.geoderma.2011.05.012
- 720 60. Jansen B, Wiesenberg GLB. Opportunities and limitations related to the application of plant-
721 derived lipid molecular proxies in soil science. *SOIL*. 2017;3: 211–234. doi:10.5194/soil-3-
722 211-2017
- 723 61. Jansen B, de Boer EJ, Cleef AM, Hooghiemstra H, Moscol-Olivera M, Tonneijck FH, et al.
724 Reconstruction of late Holocene forest dynamics in northern Ecuador from biomarkers and
725 pollen in soil cores. *Palaeogeogr Palaeoclimatol Palaeoecol*. 2013;386: 607–619.
726 doi:10.1016/j.palaeo.2013.06.027
- 727 62. Wiesenberg GLB, Andreeva DB, Chimitdorgieva GD, Erbajeva MA, Zech W. Reconstruction
728 of environmental changes during the late glacial and Holocene reflected in a soil-sedimentary
729 sequence from the lower Selenga River valley, Lake Baikal region, Siberia, assessed by lipid
730 molecular proxies. *Quat Int*. 2015;365: 190–202. doi:10.1016/j.quaint.2015.01.042
- 731 63. Lockheart MJ, van Bergen PF, Evershed RP. Chemotaxonomic classification of fossil leaves
732 from the Miocene Clarkia lake deposit, Idaho, USA based on n -alkyl lipid distributions and
733 principal component analyses. *Org Geochem*. 2000;31: 1223–1246. doi:10.1016/S0146-
734 6380(00)00107-8
- 735 64. Huang Y, Lockheart MJ, Collister JW, Eglinton G. Molecular and isotopic biogeochemistry of
736 the Miocene Clarkia Formation: hydrocarbons and alcohols. *Org Geochem*. 1995;23: 785–801.
737 doi:10.1016/0146-6380(95)80001-8
- 738 65. Poynter J, Eglinton G. Molecular Composition of Three Sediments from Hole 717C: The
739 Bengal Fan. *Proceedings of the Ocean Drilling Program, 116 Scientific Results*. Ocean
740 Drilling Program; 1990. pp. 155–161. doi:10.2973/odp.proc.sr.116.151.1990
- 741 66. Castañeda IS, Werne JP, Johnson TC, Filley TR. Late Quaternary vegetation history of
742 southeast Africa: The molecular isotopic record from Lake Malawi. *Palaeogeogr*
743 *Palaeoclimatol Palaeoecol*. 2009;275: 100–112. doi:10.1016/j.palaeo.2009.02.008
- 744 67. Rommerskirchen F, Plader A, Eglinton G, Chikaraishi Y, Rullkötter J. Chemotaxonomic
745 significance of distribution and stable carbon isotopic composition of long-chain alkanes and
746 alkan-1-ols in C4 grass waxes. *Org Geochem*. 2006;37: 1303–1332.

- 747 doi:10.1016/j.orggeochem.2005.12.013
- 748 68. Otto A, Simpson MJ. Degradation and Preservation of Vascular Plant-derived Biomarkers in
749 Grassland and Forest Soils from Western Canada. *Biogeochemistry*. 2005;74: 377–409.
750 doi:10.1007/s10533-004-5834-8
- 751 69. Han J, Calvin M. Hydrocarbon Distribution of Algae and Bacteria, and Microbiological
752 Activity in Sediments. *Proc Natl Acad Sci*. 1969;64: 436–443. doi:10.1073/pnas.64.2.436
- 753 70. Jansen B, Nierop KGJ, Hageman JA, Cleef AM, Verstraten JM. The straight-chain lipid
754 biomarker composition of plant species responsible for the dominant biomass production along
755 two altitudinal transects in the Ecuadorian Andes. *Org Geochem*. 2006;37: 1514–1536.
756 doi:10.1016/j.orggeochem.2006.06.018
- 757 71. Schäfer IK, Lanny V, Franke J, Eglinton TI, Zech M, Vysloužilová B, et al. Leaf waxes in
758 litter and topsoils along a European transect. *SOIL*. 2016;2: 551–564. doi:10.5194/soil-2-551-
759 2016
- 760 72. Franich RA, Goodin SJ, Volkman JK. Alkyl esters from *pinus radiata* foliage epicuticular wax.
761 *Phytochemistry*. 1985;24: 2949–2952. doi:10.1016/0031-9422(85)80033-9
- 762 73. Sümmechen P, Markstädter C, Wienhaus O. Composition of the Epicuticular Wax Esters of
763 *Picea abies* (L.) Karst. *Zeitschrift für Naturforsch C*. 1995;50: 11–14. doi:10.1515/znc-1995-1-
764 203
- 765 74. Koch K, Ensikat H-J. The hydrophobic coatings of plant surfaces: Epicuticular wax crystals
766 and their morphologies, crystallinity and molecular self-assembly. *Micron*. 2008;39: 759–772.
767 doi:10.1016/j.micron.2007.11.010
- 768 75. Cranwell PA, Volkman JK. Alkyl and steryl esters in a recent lacustrine sediment. *Chem Geol*.
769 1981;32: 29–43. doi:10.1016/0009-2541(81)90126-1
- 770 76. van Bergen PF, Bull ID, Poulton PR, Evershed RP. Organic geochemical studies of soils from
771 the Rothamsted Classical Experiments—I. Total lipid extracts, solvent insoluble residues and
772 humic acids from Broadbalk Wilderness. *Org Geochem*. 1997;26: 117–135.
773 doi:10.1016/S0146-6380(96)00134-9
- 774 77. Lihavainen J, Ahonen V, Keski-Saari S, Söber A, Oksanen E, Keinänen M. Low vapor

- 775 pressure deficit reduces glandular trichome density and modifies the chemical composition of
776 cuticular waxes in silver birch leaves. *Tree Physiol.* 2017;37: 1166–1181.
777 doi:10.1093/treephys/tpx045
- 778 78. Zech M, Andreev A, Zech R, Müller S, Hambach U, Frechen M, et al. Quaternary vegetation
779 changes derived from a loess-like permafrost palaeosol sequence in northeast Siberia using
780 alkane biomarker and pollen analyses. *Boreas.* 2010;39: 540–550. doi:10.1111/j.1502-
781 3885.2009.00132.x
- 782 79. Tarasov PE, Müller S, Zech M, Andreeva D, Diekmann B, Leipe C. Last glacial vegetation
783 reconstructions in the extreme-continental eastern Asia: Potentials of pollen and n-alkane
784 biomarker analyses. *Quat Int.* 2013;290–291: 253–263. doi:10.1016/j.quaint.2012.04.007
- 785 80. Berke MA, Cartagena Sierra A, Bush R, Cheah D, O'Connor K. Controls on leaf wax
786 fractionation and $\delta^2\text{H}$ values in tundra vascular plants from western Greenland. *Geochim*
787 *Cosmochim Acta.* 2019;244: 565–583. doi:10.1016/j.gca.2018.10.020
- 788 81. Mayes RW, Beresford NA, Lamb CS, Barnett CL, Howard BJ, Jones B-EV, et al. Novel
789 approaches to the estimation of intake and bioavailability of radiocaesium in ruminants grazing
790 forested areas. *Sci Total Environ.* 1994;157: 289–300. doi:10.1016/0048-9697(94)90592-4
- 791 82. Daniels WC, Russell JM, Giblin AE, Welker JM, Klein ES, Huang Y. Hydrogen isotope
792 fractionation in leaf waxes in the Alaskan Arctic tundra. *Geochim Cosmochim Acta.*
793 2017;213: 216–236. doi:10.1016/j.gca.2017.06.028
- 794 83. Ronkainen T, Väiliranta M, McClymont E, Biasi C, Salonen S, Fontana S, et al. A combined
795 biogeochemical and palaeobotanical approach to study permafrost environments and past
796 dynamics. *J Quat Sci.* 2015;30: 189–200. doi:10.1002/jqs.2763
- 797 84. Balascio NL, D'Andrea WJ, Gjerde M, Bakke J. Hydroclimate variability of High Arctic
798 Svalbard during the Holocene inferred from hydrogen isotopes of leaf waxes. *Quat Sci Rev.*
799 2018;183: 177–187. doi:10.1016/j.quascirev.2016.11.036
- 800 85. Rao SJ, Iason GR, Hulbert IA, Mayes RW, Racey PA. Estimating diet composition for
801 mountain hares in newly established native woodland: development and application of plant-
802 wax faecal markers. *Can J Zool.* 2003;81: 1047–1056. doi:10.1139/z03-093

- 803 86. Pagani M, Pedentchouk N, Huber M, Sluijs A, Schouten S, Brinkhuis H, et al. Arctic
804 hydrology during global warming at the Palaeocene/Eocene thermal maximum. *Nature*.
805 2006;442: 671–675. doi:10.1038/nature05043
- 806 87. Sachse D, Radke J, Gleixner G. δD values of individual n-alkanes from terrestrial plants along
807 a climatic gradient – Implications for the sedimentary biomarker record. *Org Geochem*.
808 2006;37: 469–483. doi:10.1016/j.orggeochem.2005.12.003
- 809 88. van den Bos V, Engels S, Bohncke SJP, Cerli C, Jansen B, Kalbitz K, et al. Late Holocene
810 changes in vegetation and atmospheric circulation at Lake Uddelermeer (The Netherlands)
811 reconstructed using lipid biomarkers and compound-specific δD analysis. *J Quat Sci*. 2018;33:
812 100–111. doi:10.1002/jqs.3006
- 813 89. Dawson LA, Towers W, Mayes RW, Craig J, Väisänen RK, Waterhouse EC. The use of plant
814 hydrocarbon signatures in characterizing soil organic matter. *Geol Soc London, Spec Publ*.
815 2004;232: 269–276. doi:10.1144/GSL.SP.2004.232.01.24
- 816 90. Huang Y, Eglinton G, Ineson P, Bol R, Harkness DD. The effects of nitrogen fertilisation and
817 elevated CO₂ on the lipid biosynthesis and carbon isotopic discrimination in birch seedlings
818 (*Betula pendula*). *Plant Soil*. 1999;216: 35–45. doi:10.1023/A:1004771431093
- 819 91. Maffei M, Badino S, Bossi S. Chemotaxonomic significance of leaf wax n-alkanes in the
820 Pinales (Coniferales). *J Biol Res (Thessaloniki, Greece)*. 2004;1: 3–19. Available:
821 <http://www.auth.gr/jbr/papers20041/01-2004.pdf>
- 822 92. Rajčević N, Janačković P, Dodoš T, Tešević V, Marin PD. Biogeographic Variation of Foliar
823 n- Alkanes of *Juniperus communis* var . *saxatilis* Pallas from the Balkans. *Chem Biodivers*.
824 2014;11: 1923–1938. doi:10.1002/cbdv.201400048
- 825 93. Ali HAM, Mayes RW, Hector BL, Verma AK, Ørskov ER. The possible use of n-alkanes,
826 long-chain fatty alcohols and long-chain fatty acids as markers in studies of the botanical
827 composition of the diet of free-ranging herbivores. *J Agric Sci*. 2005;143: 85–95.
828 doi:10.1017/S0021859605004958
- 829 94. Aichner B, Herzsuh U, Wilkes H. Influence of aquatic macrophytes on the stable carbon
830 isotopic signatures of sedimentary organic matter in lakes on the Tibetan Plateau. *Org*

- 831 Geochem. 2010;41: 706–718. doi:10.1016/j.orggeochem.2010.02.002
- 832 95. Hoffmann B, Kahmen A, Cernusak LA, Arndt SK, Sachse D. Abundance and distribution of
833 leaf wax n-alkanes in leaves of Acacia and Eucalyptus trees along a strong humidity gradient
834 in northern Australia. Org Geochem. 2013;62: 62–67. doi:10.1016/j.orggeochem.2013.07.003
- 835 96. Eley YL, Hren MT. Reconstructing vapor pressure deficit from leaf wax lipid molecular
836 distributions. Sci Rep. 2018;8: 3967. doi:10.1038/s41598-018-21959-w
- 837 97. Tipple BJ, Pagani M. Environmental control on eastern broadleaf forest species' leaf wax
838 distributions and d/h ratios. Geochim Cosmochim Acta. 2013;111: 64–77.
839 doi:10.1016/j.gca.2012.10.042
- 840 98. Kirkels FMSA, Jansen B, Kalbitz K. Consistency of plant-specific n- alkane patterns in
841 plaggen ecosystems: A review. The Holocene. 2013;23: 1355–1368.
842 doi:10.1177/0959683613486943
- 843 99. Elkington TT. Introgressive hybridization between *Betula nana* L. and *B. pubescens* Ehrh. in
844 North-West Iceland. New Phytol. 1968;67: 109–118. doi:10.1111/j.1469-8137.1968.tb05459.x
- 845 100. Palme AE, Su Q, Palsson S, Lascoux M. Extensive sharing of chloroplast haplotypes among
846 European birches indicates hybridization among *Betula pendula*, *B. pubescens* and *B. nana*.
847 Mol Ecol. 2004;13: 167–178. doi:10.1046/j.1365-294X.2003.02034.x
- 848 101. Stammitti L, Derridj S, Garrec JP. Leaf epicuticular lipids of *Prunus laurocerasus*: importance
849 of extraction methods. Phytochemistry. 1996;43: 45–48. doi:10.1016/0031-9422(96)00241-5

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852 Supporting information

853 **S1 Figure.** Total ion chromatogram of a *Betula humilis* leaf with the most abundant components.

854 **S2 Figure.** Mass spectrum of a C₄₄ alkyl ester mixture (RCOOR') of *Betula humilis*. The
855 diagnostic ions are shown for the acid fragments (RCO₂H₂⁺) and the molecular ion (M⁺).

856

857 **S3 Table**

Ester chain length	M ⁺	Mass (μg/g d.w.)	Acid chain length	Alcohol chain length	Amount of isomer (μg/g d.w.)
38	564	3.06	14	24	0.78
			16	22	2.28
40	592	17.1	14	26	1.05
			16	24	10.0
			18	22	4.95
			20	20	1.20
42	620	58.9	14	28	1.73
			16	26	11.8
			18	24	22.9
			20	22	21.9
			22	20	0.16
			24	18	0.34
44	648	104.2	14	30	0.60
			16	28	14.6
			18	26	14.4
			20	24	63.4
			22	22	10.4
			24	20	0.78
46	676	52.8	14	32	0.13
			16	30	1.99
			18	28	8.54
			20	26	27.9
			22	24	11.4
			24	22	2.86
48	704	22.1	20	28	16.1
			22	26	2.35
			24	24	3.62

S4 Table

Ester chain length	M+	Mass ($\mu\text{g/g}$ d.w.)	Acid chain length	Alcohol chain length	Amount of isomer ($\mu\text{g/g}$ d.w.)
36	536	1047	14	22	6.5
			16	20	1040
38	564	601	14	24	7.5
			16	22	420
			18	20	173
40	592	532	14	26	3.0
			16	24	254
			18	22	123
			20	20	150
42	620	310	14	28	2.4
			16	26	57.7
			18	24	57.9
			20	22	147
			22	20	44.7
44	648	180	16	28	39.7
			18	26	10.9
			20	24	59.2
			22	22	49.3
			24	20	21.1
46	676	70	16	30	6.9
			18	28	5.6
			20	26	10.2
			22	24	16.6
			24	22	11.3
			26	20	19.8
48	704	35	16	32	3.62
			20	28	6.03
			24	24	4.95
			26	22	6.18
			28	20	14.2

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S5 Table

Ester chain length	M+	Mass ($\mu\text{g/g d.w.}$)	Acid chain length	Alcohol chain length	Amount of isomer ($\mu\text{g/g d.w.}$)
38	564	535.74	14	24	13.6
			16	22	519
			18	20	2.73
			20	18	0.57
40	592	290.49	14	26	7.29
			16	24	208
			18	22	71.5
			20	20	4.06
42	620	147.36	14	28	4.85
			16	26	67.3
			18	24	22.8
			20	22	52.4
44	648	68.44	16	28	39.7
			18	26	9.65
			20	24	19.0

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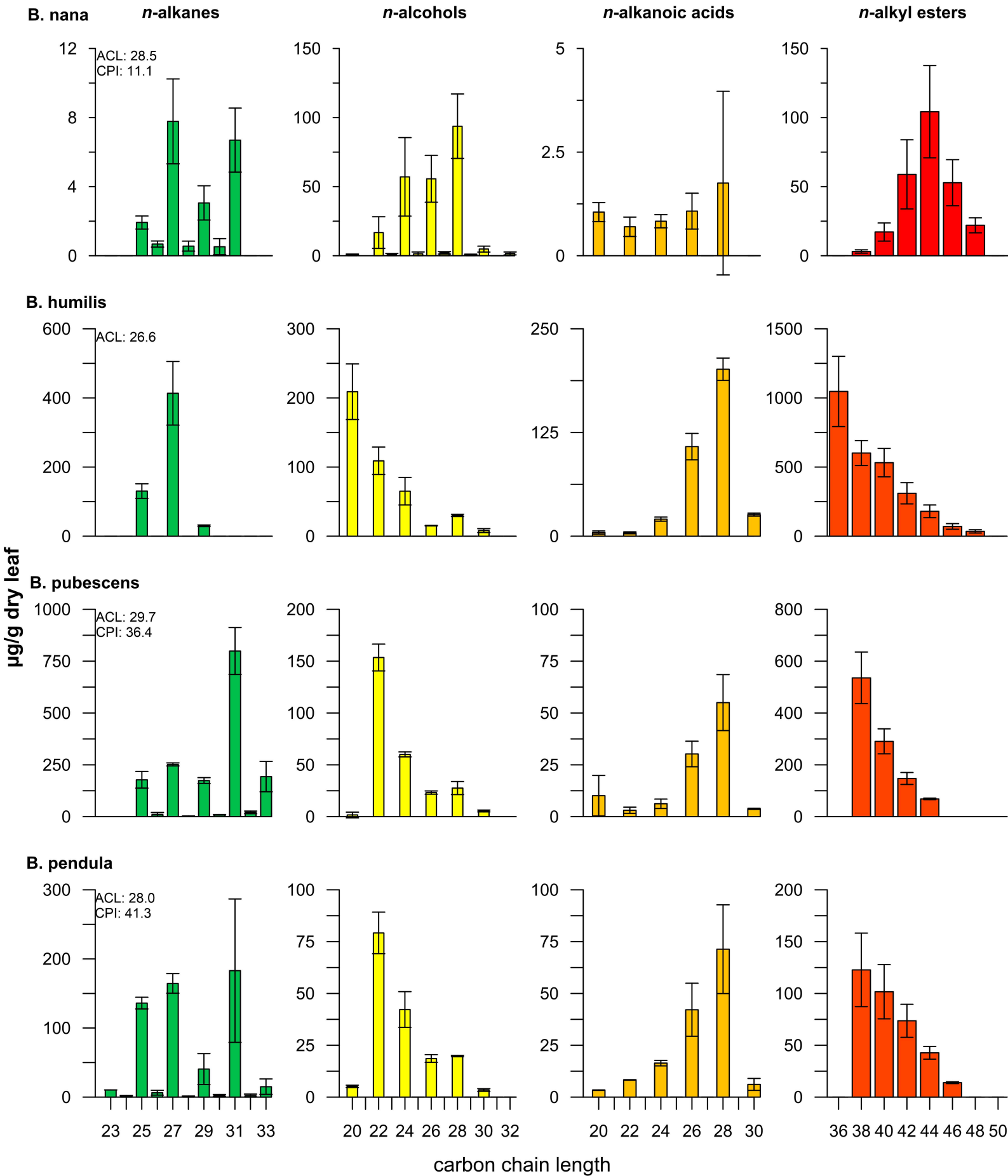
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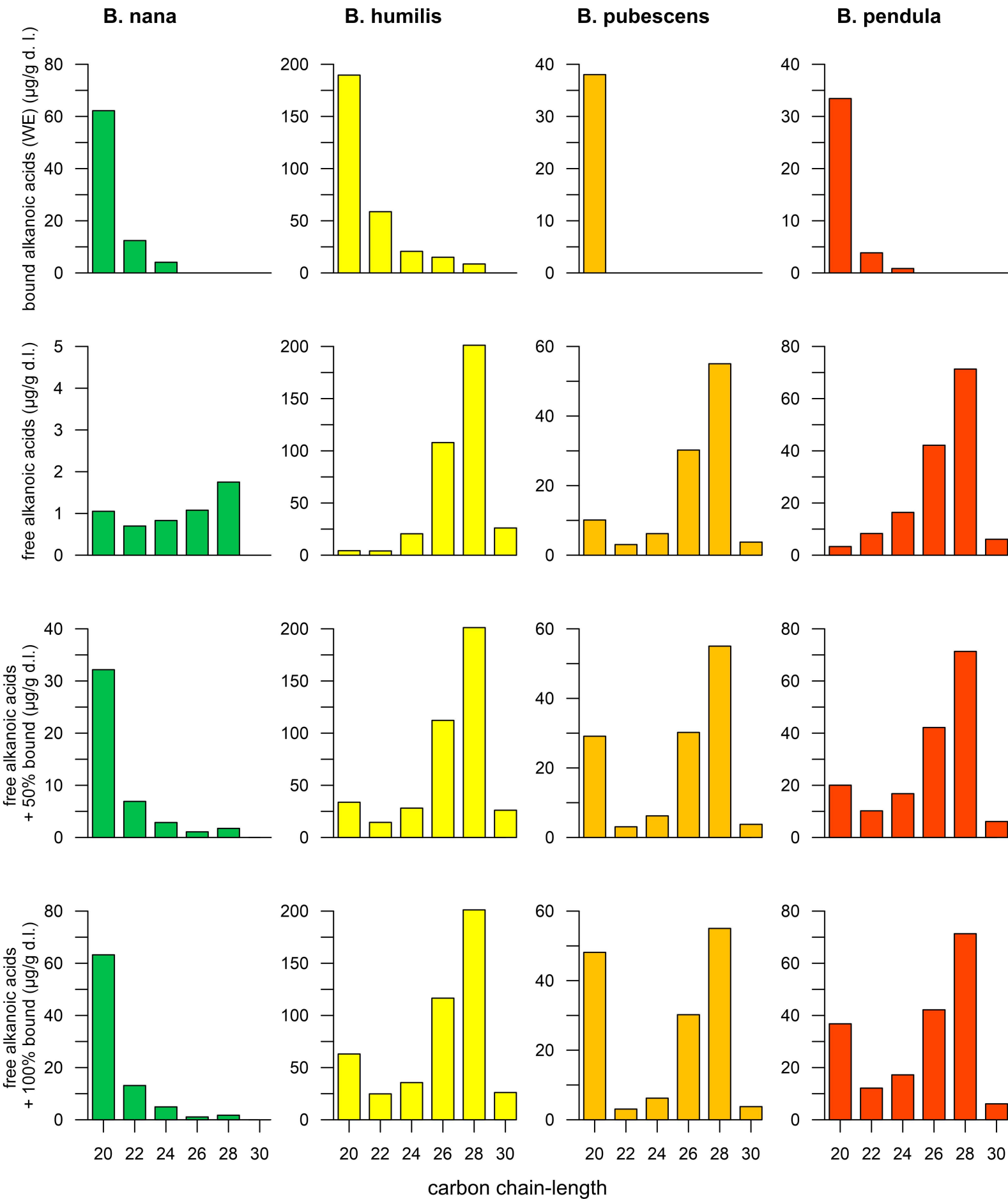
S6 Table

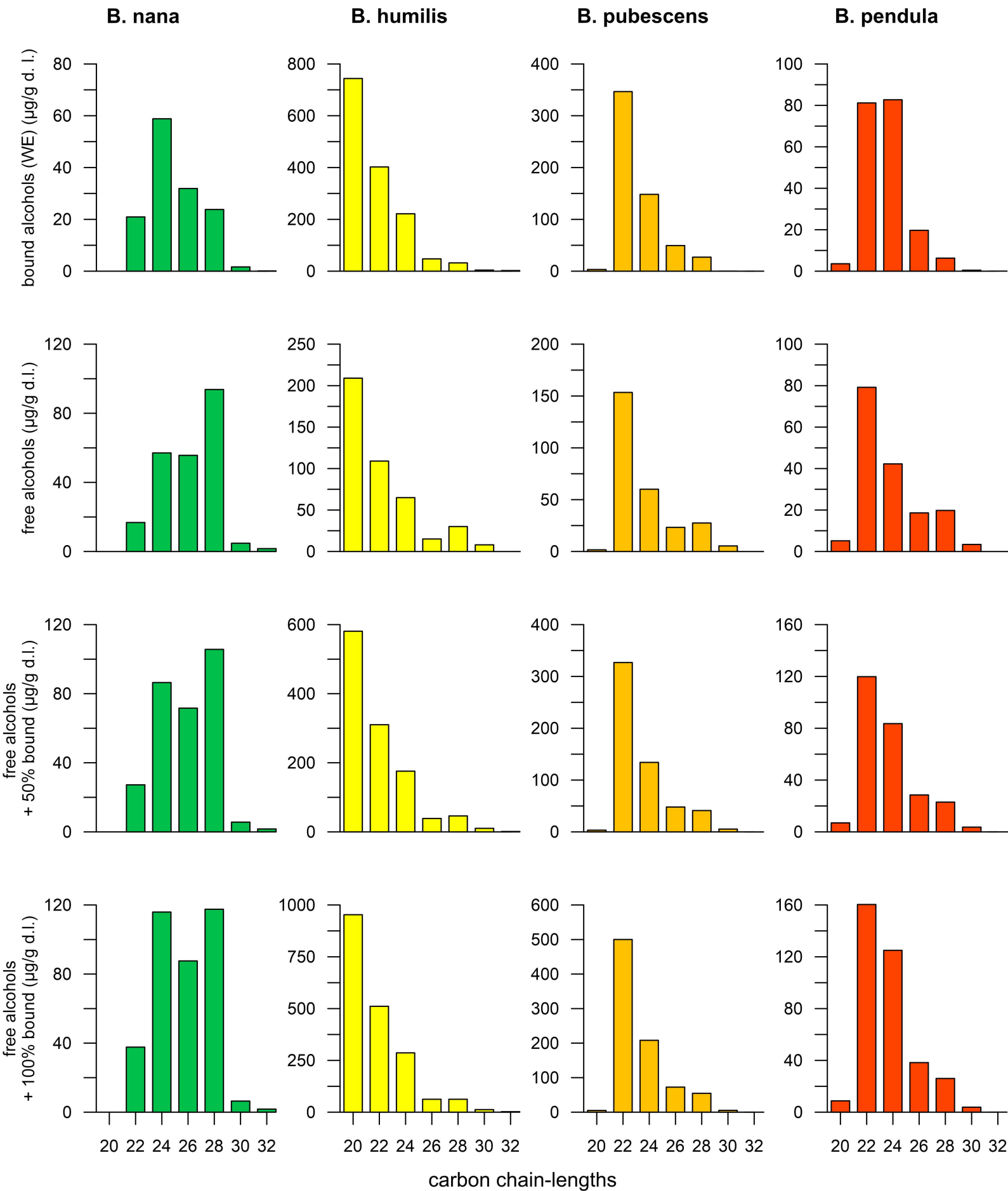
Ester chain length	M+	Mass (µg/g d.w.)	Acid chain length	Alcohol chain length	Amount of isomer (µg/g d.w.)
38	564	122.7	14	24	31.3
			16	22	91.5
40	592	101.7	14	26	6.20
			16	24	59.1
			18	22	29.3
			20	20	7.08
42	620	73.6	14	28	2.16
			16	26	14.7
			18	24	28.7
			20	22	27.4
			22	20	0.20
			24	18	0.42
44	648	42.7	14	30	0.25
			16	28	5.98
			18	26	5.92
			20	24	26.0
			22	22	4.26
			24	20	0.32
46	676	13.9	14	32	0.03
			16	30	0.52
			18	28	2.25
			20	26	7.36
			22	24	3.01
			24	22	0.75

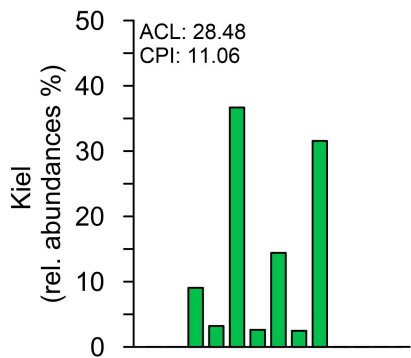
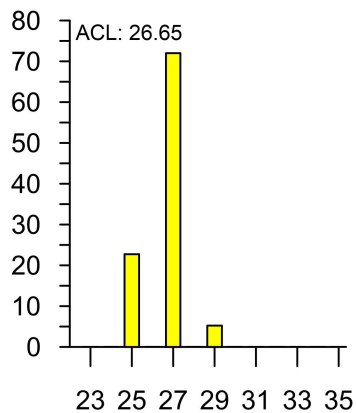
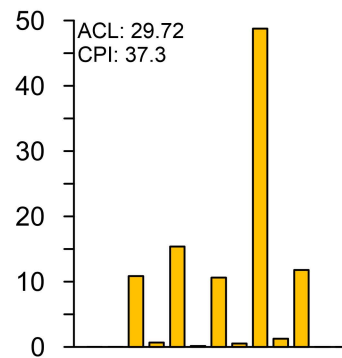
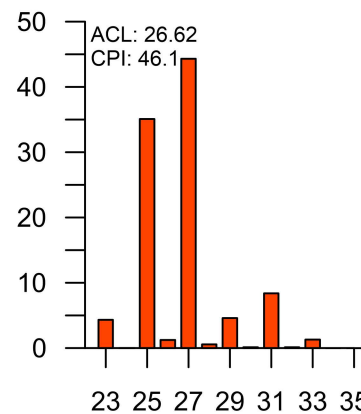
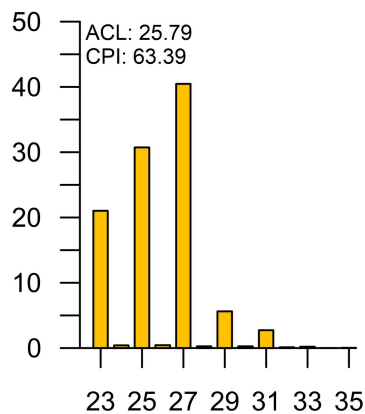
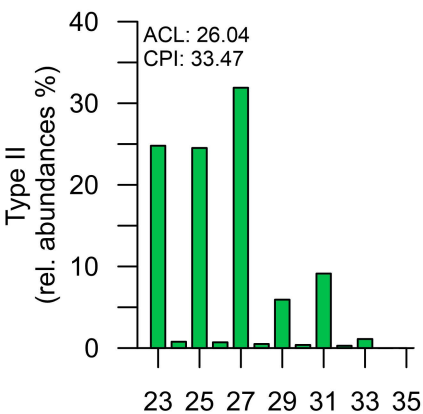
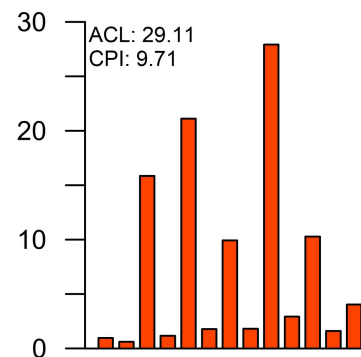
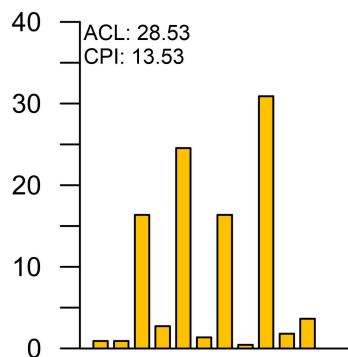
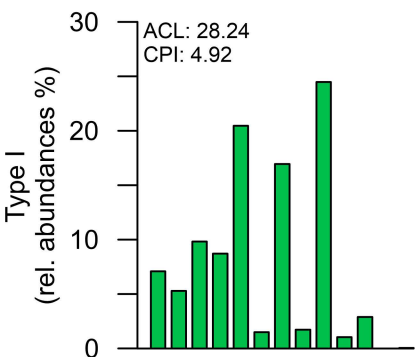
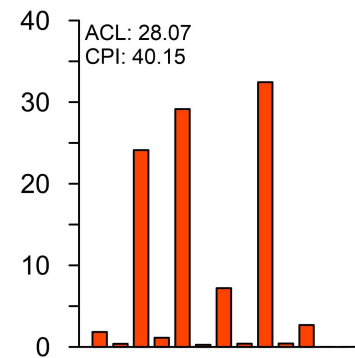
875

876



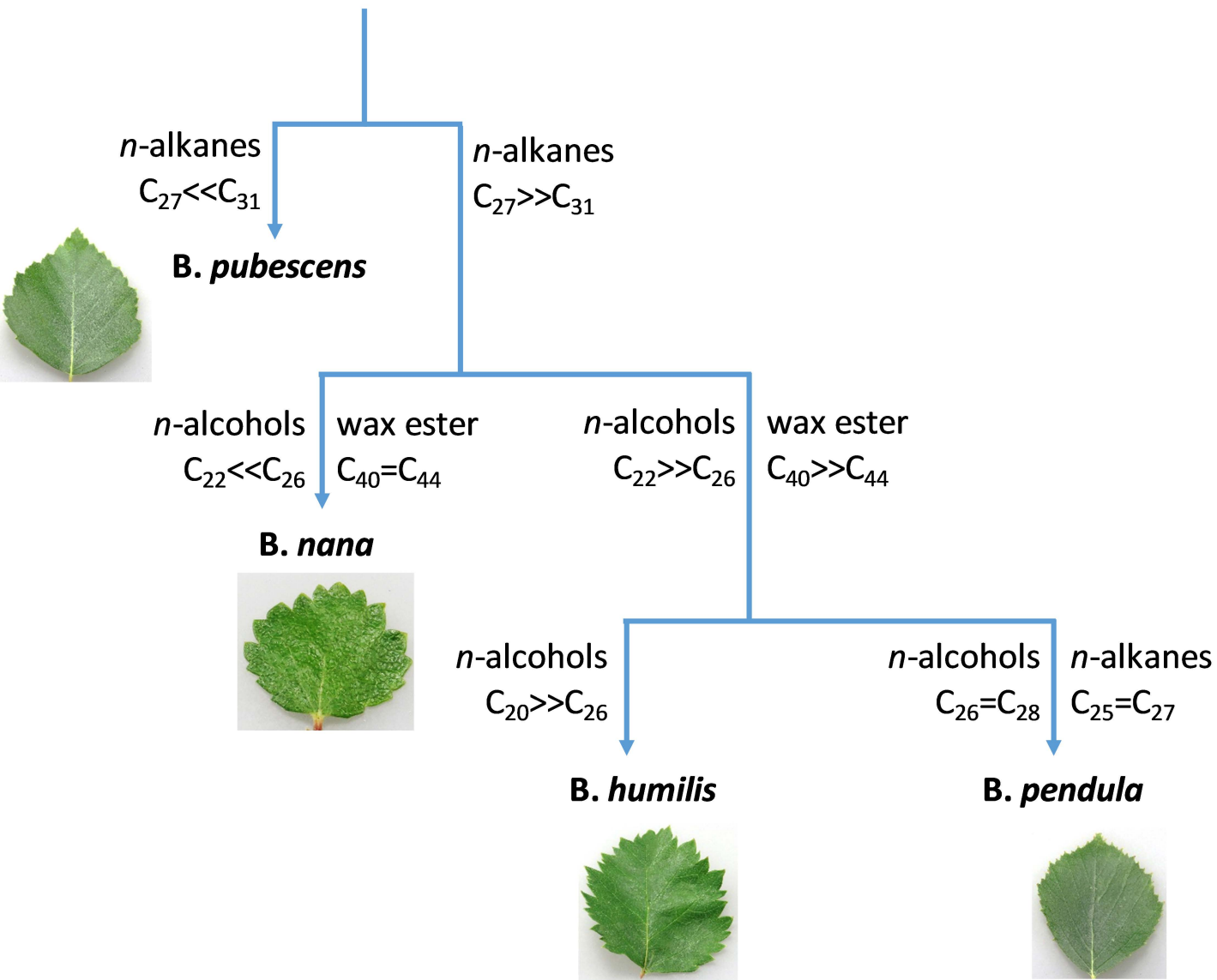




B. nana**B. humilis****B. pubescens****B. pendula**

carbon chain-lengths

wax lipid discrimination tree



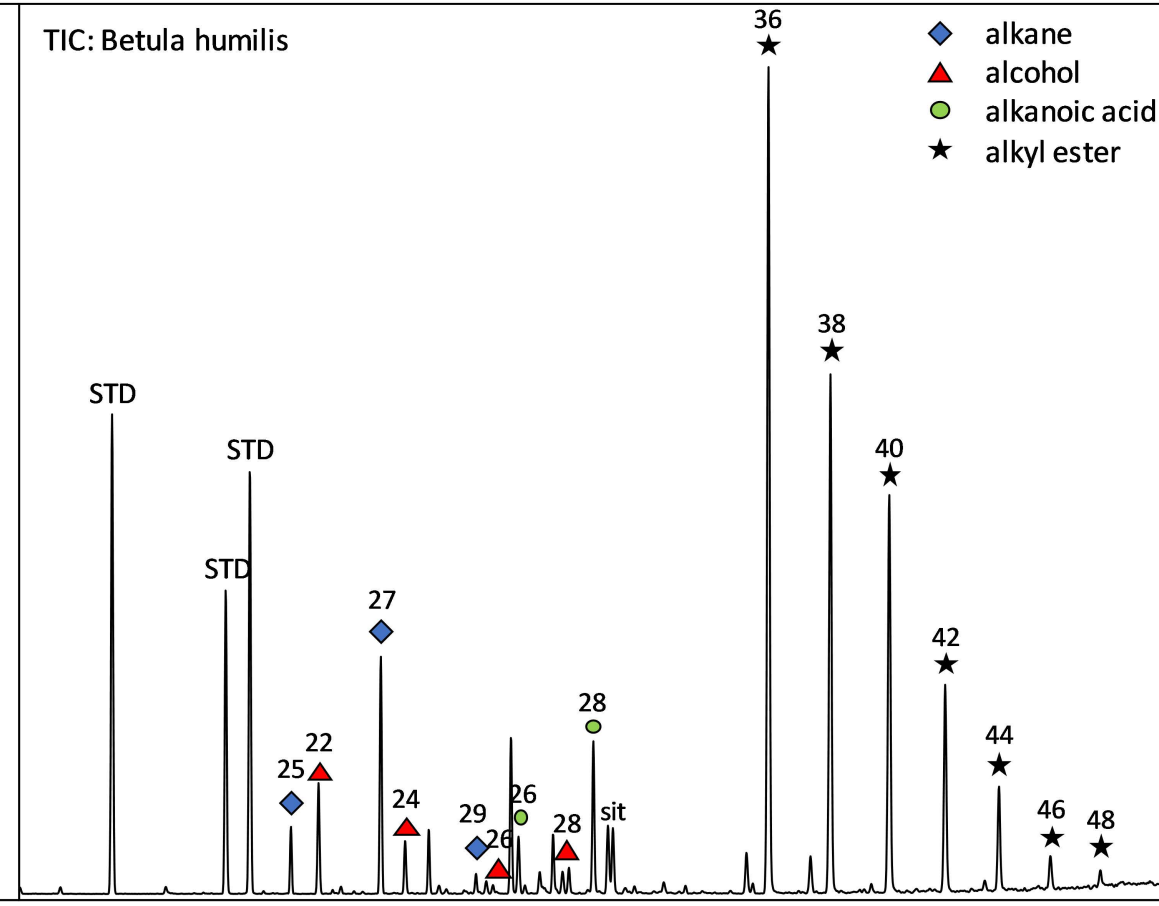
100

TIC: *Betula humilis*

- ◆ alkane
- ▲ alcohol
- alkanolic acid
- ★ alkyl ester

Relative abundance (%)

0



100

WE 44

313

Relative abundance (%)

341

257

369

57

97

139

229

285

M+
648

0

50

100

150

200

250

300

350

400

450

500

550

600

650