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ω20-Hydroxy and ω9,ω10-dihydroxy biomarker lipids in ferns from the Salviniaceae family

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ABSTRACT

All seven species of floating ferns from the genus *Azolla* (family Salviniaceae) produce a unique series of long chain mid-chain ω20-hydroxy compounds (ω20-alkanols, 1,ω20-diols, ω20-hydroxy fatty acids) and structurally related ω9,ω10-dihydroxy compounds (ω9,ω10-diols, 1,ω9,ω10-triols and ω9,ω10-dihydroxy fatty acids). These very long chain fatty acid (VLCFA) derivatives occur in the ferns' waxes in free and esterified form. The specific distribution of these lipids differed between species belonging to each of the two sections in the *Azolla* genus: in species of the section *Azolla* and *Rhizosperma,* the ratio of C_{31} over C_{35} ω20-alkanols averaged 7.0 and 0.40, and the ratio of C_{26} over C_{28} ω20-hydroxy fatty acids averaged 2.7 and 1.0, respectively. Similar compounds were identified in species of another genus in the Salviniaceae family, *Salvinia,* suggesting that their biosynthetic pathway evolved early during Salviniaceae evolution (>89 Ma). *Salvinia* species contain ω20-hydroxy and ω9,ω10-dihydroxy compounds in smaller concentrations and in a much different distribution compared to *Azolla*; the C_{31} 1, ω20-diol is unique to *Salvinia* species. Closely related fern species from the genera *Marsilea, Pilularia* and *Regnellidium* did not contain these compounds, nor did unrelated aquatic plants from the genera *Lemna* and *Pistia.* All mid-chain hydroxy compounds detected in extant *Azolla* have been traced previously in Arctic Eocene sediments from the so-called 'Azolla Event' (48.5 Ma), implying that they are well preserved in the geological record and may therefore serve as *Azolla* biomarkers. Our findings indicate that ω20-hydroxy and ω9,ω10-dihydroxy compounds in sediments could be used as biomarkers of the whole Salviniaceae family. Subsequently, the clear differences in compound distribution between the *Azolla* and *Salvinia* genera and the more subtle ones between the two *Azolla* sections, may allow assigning the compound's origin at the genus (and possibly section) level, depending on the preservation of compound classes in the sediment and the timing of the *Azolla* or *Salvinia* deposition. This is exemplified by a sediment interval of the so-called '*Salvinia* bed' (Eemian), which contained trace amounts of the C_{31} 1, ω20-diol, but none of the ω20-hydroxy and ω9,ω10-dihydroxy compounds common to *Azolla*, indicating the value of C_{31} 1,ω20-diol as a biomarker for distinguishing *Salvinia* from *Azolla*.

1. Introduction

Highly laminated Eocene sediments from the central Arctic Ocean, IODP expedition 302, have been found to contain intact microspore massulae and megaspores of the free-floating aquatic fern *A. arctica* sp. nov., indicating that this *Azolla* species grew, reproduced and bloomed in situ during an extended period of ~1.2 Myr (Brinkhuis et al., 2006; Collinson et al., 2009; Speelman et al., 2009b) roughly 48.5 Ma. The occurrence of *Azolla* spp. in the Eocene Arctic Ocean setting is remarkable, since extant *Azolla* such as *A. filiculoides*, cannot tolerate salinities higher than 1–1.6 g/kg (e.g., Rai and Rai, 1999) and for that reason, its presence in sediments may serve as an important indicator of episodes of past freshwater-dominated environments (Brinkhuis et al., 2006; Speelman et al., 2009a).

Azolla is a genus of aquatic ferns consisting of two sections (*Azolla* and *Rhizosperma*) which together comprise seven different species. The section *Azolla* is believed to consist of five species:

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A. filiculoides, A. rubra, A. caroliniana, A. mexicana, and *A. microphylla*. The species *A. rubra*, however, may be classified as a variety of *A. filiculoides* (Wagner, 1997). Similarly, the taxonomic status and rank of three other taxa (*A. caroliniana, A. mexicana*, and *A. microphylla*) have also generated considerable debate (Metzgar et al., 2007). The *Rhizosperma* section contains *A. pinnata* and *A. nilotica*; the third species initially assigned to this section, *A. imbricata*, was later recognized as a variety of *A. pinnata* (Shen, 1961; Sweet and Hills, 1971). The two *Azolla* sections diverged during the Eocene (~50 Ma) (Metzgar et al., 2007), and the *Azolla* genus deviated from its most closely related genus *Salvinia* already during the Cretaceous (89 Ma) (Pryer et al., 2004).

A. filiculoides was found to produce several unique series of long chain mid-chain (ω20) hydroxy compounds: odd numbered ω20-alkanols, even numbered 1,ω20-diols, and even numbered ω20-hydroxy fatty acids, all of which range within C_{25}–C_{36} (Speelman et al., 2009a). Palmitate ($C_{16:0}$) esters of 1,ω20-diols were also identified. Additionally, several structurally related ω9,ω10-dihydroxy compounds, predominantly as C_{29} members (20,21-dihydroxy compounds), were identified as well as their palmitate wax esters (Speelman et al., 2009a) (Fig. 1). Similarly, both *A. microphylla* and *A. pinnata* (var. *imbricata*) were shown to contain these (di)hydroxy lipids (Atwood et al., 2014; Mao et al., 2017). These compounds have also been detected in recent sediments (up to 9.1 kyr BP) in El Junco Lake (Galápagos) (Zhang et al., 2011; Atwood et al., 2014) and in Arctic Eocene sediments (Speelman et al., 2009a), implying that they are relatively resistant to degradation.

Based on the unique occurrence and stability in geological settings of the ω20-hydroxy and ω9,ω10-dihydroxy compounds, it has been proposed that they could serve as prime proxies for *Azolla* spp. in paleoarchives, such as sediments. Whether these compounds are similarly distributed in all known *Azolla* species remains unknown, however. The claim for specificity has also not been tested yet as their occurrence in ferns from closely related fern genera, including *Salvinia* and *Marsilea*, *Pilularia* and *Regnellidium* has not been explored. Furthermore, the capability to produce these compounds may have specific advantages in an aquatic habitat, and therefore could have evolved in parallel and independently in non-related aquatic species.

To validate their use as biomarkers, we investigated the occurrence and distribution of the ω20-hydroxy and ω9,ω10-dihydroxy compounds in all species of the *Azolla* genus, fern genera closely related to *Azolla* and non-related seed plants that share its habitat. Ten different *Azolla* strains were examined including at least one strain for each of the seven *Azolla* species as well as the *A. pinnata* variety *imbricata* (referred to as *A. imbricata* after this). Two strains of *A. filiculoides* and *A. nilotica* were included to evaluate intraspecies variability. Secondly, we investigated a set of representative species of closely related fern genera: *Salvinia, Marsilea*, *Pilularia* and *Regnellidium*. The floating aquatic plants *Lemna trisulca* and *Pistia stratiotes* were examined as non-related genera, as they are often found to share their habitat with *Azolla* species (Drago, 2007; Johnston et al., 2007).

2. Materials and methods

2.1. Plant collection

A. filiculoides Wild Type (WT) was collected from a pond near Galgenwaard, Utrecht, The Netherlands. All other *Azolla* specimens were obtained from the International Rice Research Institute (IRRI), Philippines, under accession numbers 535 (*A. imbricata*; originally from Sigiriya, Sri Lanka), 1052 (*A. filiculoides*; originally from Lyon, France), 2007 (*A. mexicana*; originally from C. Kettering lab, USA), 3504 (*A. caroliniana*; originally from Solimoes River, Brazil), 4059 (*A. microphylla*; originally from South Cotobato, Philippines), 5002 and 5501 (both *A. nilotica* 1 and 2; originally from Kosti, Sudan, and Bujumbura, Burundi, respectively), 6502 (*A. rubra*; originally from Victoria, Australia), and 7004 (*A. pinnata*; originally from Fog Dam, Australia) (Watanabe et al., 1992). *Lemna trisulca* was obtained from a plant nursery in Utrecht, The Netherlands. *Salvinia molesta, S. minima, S. oblongifolia, S. cucullata* were supplied by the Bonn University Botanic Gardens, Germany. *Salvinia* sp., *Mar-*

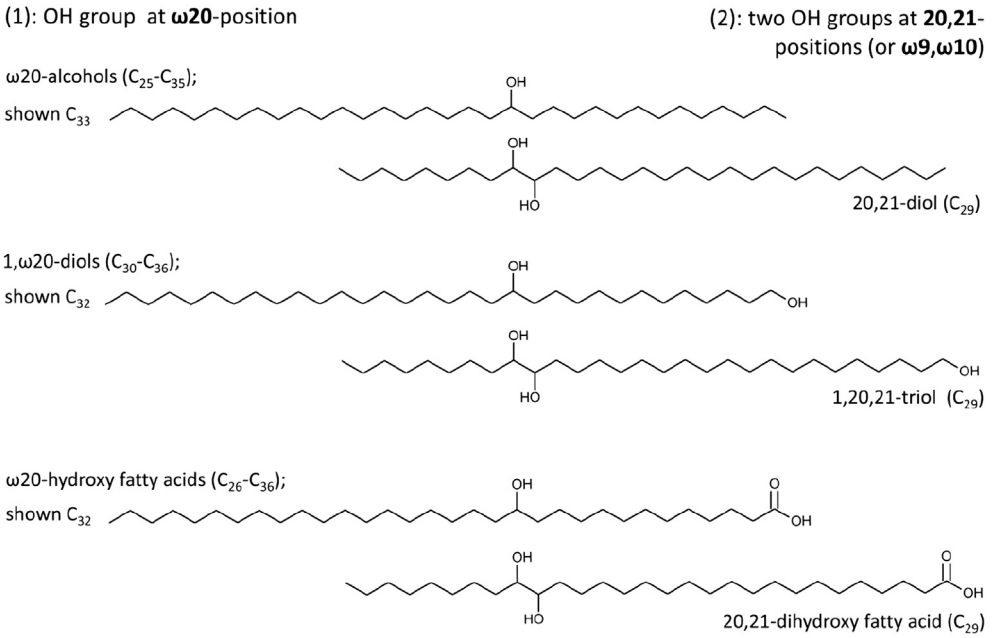

Fig. 1. Structures and chain length distribution of the ω20-hydroxy and ω9,ω10-dihydroxy compounds identified in *Azolla*. From top to bottom they represent ω20-hydroxylated and ω9,ω10-dihydroxylated *n*-alkanes, 1-alkanols and fatty acids, respectively.

silea quadrifolia, *M. hirsuta, Pilularia globulifera, Regnellidium diphyllum* and *Pistia stratiotes* were obtained from the Utrecht Botanic Gardens, The Netherlands. All these plants were analysed as a whole plant, except for *S. oblongifolia*, for which the plants were separated into leaves and roots, and the *Marsileaceae* species, of which only the leaves were analysed. In order to determine the lipid composition of various *Azolla* plant tissues, leaves, roots, megaspores and microspore massulae of *A. filiculoides* (WT) were isolated and subsequently analysed similar to whole plants. Prior to analysis all plant specimens were either air-dried (at 30 °C) or freeze-dried.

Sediments from the Kreftenheye Formation, which contains a so-called *Salvinia* bed, were used to trace possible markers of *Salvinia* in the geological record (Van der Ham et al., 2008). Air-dried samples were a gift from Jan Brewer (Raalte) and were crushed prior to biomarker extraction.

2.2. Extraction and work-up

The freeze-dried plants and tissues were powdered and subsequently extracted using dichloromethane (DCM)-methanol (MeOH) mixtures. Extraction was ultrasonically performed in DCM:MeOH (2:1, v/v; 5 × 10 min). due to the small amounts (50–100 mg) available, with the exception of all *Salvinia* species, the *Salvinia* bed sediments, and *A. filiculoides* WT, which were available in larger quantities (>0.5 g) and were Soxhlet extracted in DCM:MeOH (9:1, v/v, 24 h). The total lipid extracts (TLEs) obtained were dried over Na_2SO_4 followed by evaporation of the solvent under a gentle stream of N_2. For TLE analyses of the aquatic plant specimens, aliquots of the TLEs were methylated with diazomethane to convert acid groups into corresponding methyl esters, purified over a SiO_2 column, and silylated using *bis*(trimethylsilyl)trifluoracetamide (BSTFA) in pyridine at 60 °C for 20 min to convert hydroxy groups into the corresponding trimethylsilyl (TMS) ethers.

A second aliquot of the TLEs was saponified in 1 M KOH in 96% MeOH for 2 h at 70 °C. After cooling, the reaction mixture was acidified using 2 M HCl in MeOH:water (1:1, v/v). The mixture was successively washed 3–5 times with DCM until the DCM phase became colourless, after which all DCM fractions were combined and dried over Na_2SO_4. After removal of the solvent under a gentle stream of N_2, the saponified extracts (SEs) were derivatised in the same way as the initial TLEs as described above.

Known amounts of squalane were added as an internal standard to the TLEs for quantification. For the SEs the internal standard was added prior to saponification to correct for possible losses during the work-up procedure and to allow a reliable comparison between intact and hydrolysed lipids. Prior to analysis on GC-FID and GC–MS all aliquots were dissolved in ethyl acetate.

2.3. Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS)

TLEs and SEs were on-column injected on a Hewlett Packard gas chromatograph-flame ionization detector (GC-FID) and a Thermo Trace GC Ultra Trace DSQ GC–MS (Thermo Fisher Scientific Inc.) using a CP-sil 5CB fused silica column (30 m × 0.32 mm i.d., 0.10 μm film thickness). The GC-FID was operated at constant pressure of 100 kPa, whereas the GC–MS was operated at constant flow of 1.0 mL/min. The oven of both GC's was programmed starting at 70 °C to rise to 130 °C at a rate of 20 °C/min and then to 320 °C at a rate of 4 °C/min, followed by an isothermal hold for 20 min. The MS was operated in full data acquisition mode, scanning ions from *m/z* 50 to 800. Identification of compounds was based on mass spectra published by Speelman et al. (2009a) and Brouwer et al. (2016).

2.4. Data analysis

The chain-length distribution of ω20-alkanols, 1,ω20-diols and ω20-hydroxy FAs for the ten *Azolla* strains was used to perform a clustering analysis. A k-mer (k = 2) clustering was performed using the IBM SPSS statistical software package. An ANOVA was performed to determine significant differences between the proposed clusters for each chain length. In case significant differences were found, the two chain lengths with the highest F-value were used to compute a ratio.

3. Results and discussion

3.1. Overall lipid composition in Azolla

The major lipids in *Azolla* include a series of fatty acids (in particular $C_{16:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$), chlorophyll, which yielded phytol after saponification, and 24-ethylcholesterol (sitosterol). In all *Azolla* strains the six major (di)hydroxy compound classes, i.e. ω20-alkanols, 1,ω20-diols, ω20-hydroxy fatty acids, ω9,ω10-diols, 1,ω9,ω10-triols, and ω9,ω10-dihydroxy fatty acids were identified. In addition, C_{30} and C_{32} 1,ω20,ω21-triols were identified after saponification. Speelman et al. (2009a) also found the ester of C_{16} fatty acid and C_{30} triol, released in small amounts after hydrolysis. Due to their very much lower concentrations compared to any of the other six compounds, we chose to not include triols in Fig. 1 and do not discuss them further.

In free form, the ω20-alkanols and the two diol types, 1,ω20-diols and ω9,ω10-diols, were the most abundant hydroxy compounds. For most species, the 1,ω20-diols comprised the largest group; only for *A. rubra* and the two *A. nilotica* species did the ω9,ω10-diols represent the dominant compounds. In contrast, after saponification, either 1,ω20-diols (*A. pinnata, A. imbricata, A. nilotica* 1 and 2 and *A. rubra*), ω20-hydroxy fatty acids (*A. filiculoides*) or 1,ω9,ω10-triols (*A. mexicana, A. microphylla* and *A. caroliniana*) were the most abundant and dominant esterified (di) hydroxy compounds (Fig. 2).

Summed together, the mid-chain hydroxy lipids ranged from 1800 μg/g dry weight (dw) in *A. mexicana* to 5100 μg/g dw in *A. nilotica*1 in TLEs. After saponification, the amounts increased, ranging from 5200 μg/g dw in *A. filiculoides* to 15,600 μg/g dw in *A. nilotica* 1.

3.2. ω20-Hydroxy compounds in Azolla species

A series of ω20-alkanols in the range C_{25}–C_{35} was identified in the various TLEs from extant *Azolla* strains with an odd-over even predominance (Fig. 3). Even-numbered ω20-alkanols of differing chain lengths were detected in trace or small amounts depending on the species: C_{28} in *A. filiculoides*, *A. nilotica* 1 and *A. rubra*, C_{30} in *A. rubra*, C_{32} in *A. rubra*, *A. caroliniana* and *A. microphylla*, and C_{34} in *A. rubra.* The concentrations of ω20-alkanols were lowest in *A. mexicana, A. microphylla* and *A. caroliniana*, whereas they were highest in *A. nilotica* 1 and, particularly, *A. pinnata*. The ω20-alkanol chain length distributions differed between the *Azolla* species: the C_{31} ω20-alkanol was strikingly low, whilst C_{35} ω20-alkanol was more abundant in species belonging to the *Rhizosperma* section (*A. pinnata, A. imbricata* and both *A. nilotica* strains) compared to the *Azolla* section. The ω20-alkanol chain length distributions were similar in TLEs and SEs, but compounds with the lowest concentrations became undetectable after saponification likely due to 'overshadowing' by other compounds.

The 1,ω20-diols identified in TLEs from extant *Azolla* species were in the range C_{30}–C_{36} and included only even-numbered members (Fig. 4). Similar to the ω20-alkanols, *A. microphylla* and

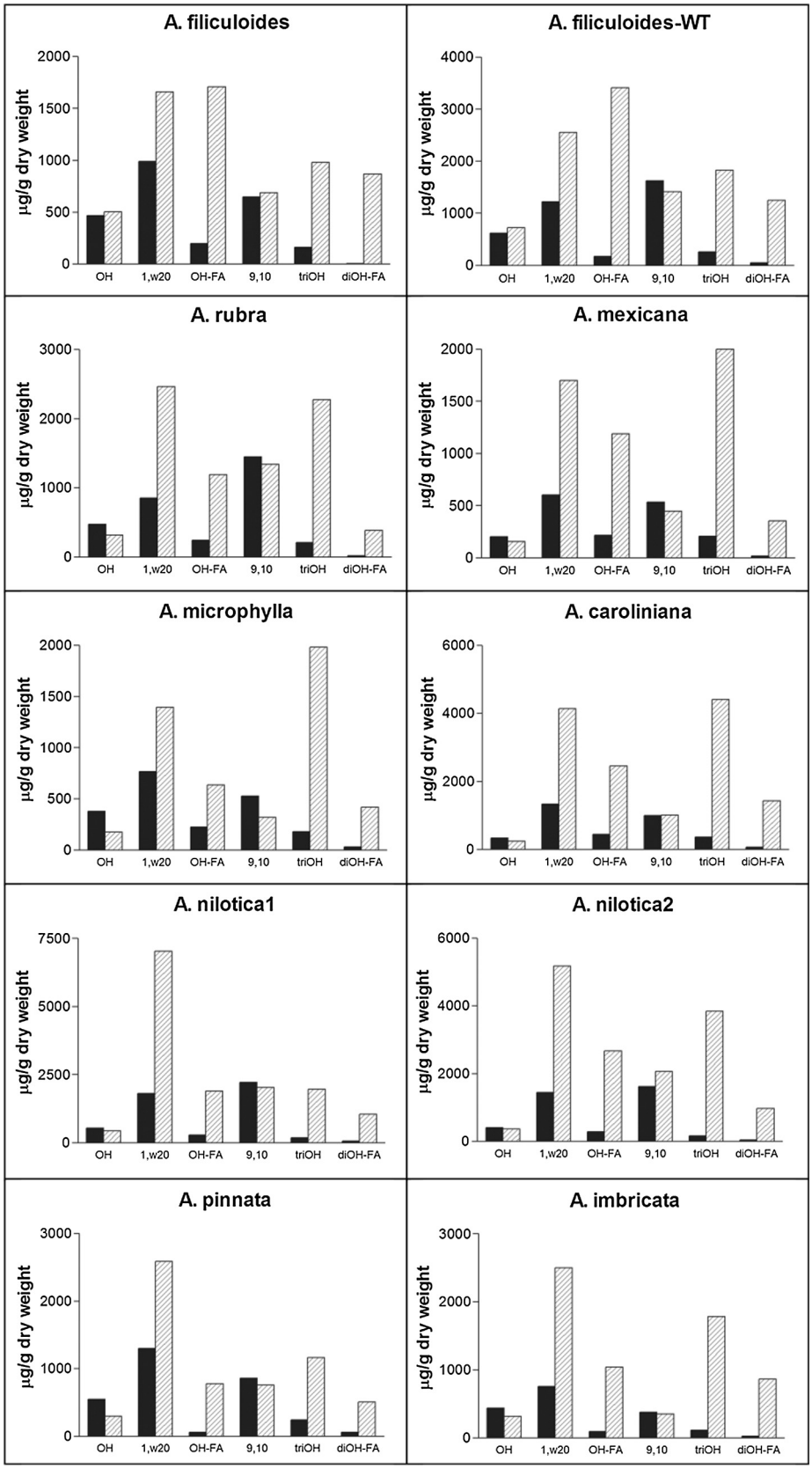

Fig. 2. Distribution of the six most abundant ω20-hydroxy and ω9,ω10-dihydroxy compounds in total lipid extracts (black bars) and saponified extracts (grey scaled bars) from different *Azolla* species. Abbreviations: OH: ω20-alkanols; 1,ω20: 1,ω20-diols; HO-FA: ω20-hydroxy fatty acids; 9,10: ω9,ω10-diols; triOH: 1,ω9,ω10-triols; and diOH-FA: ω9,ω10-dihydroxy fatty acids. Data represent sums of compounds with differing chain lengths.

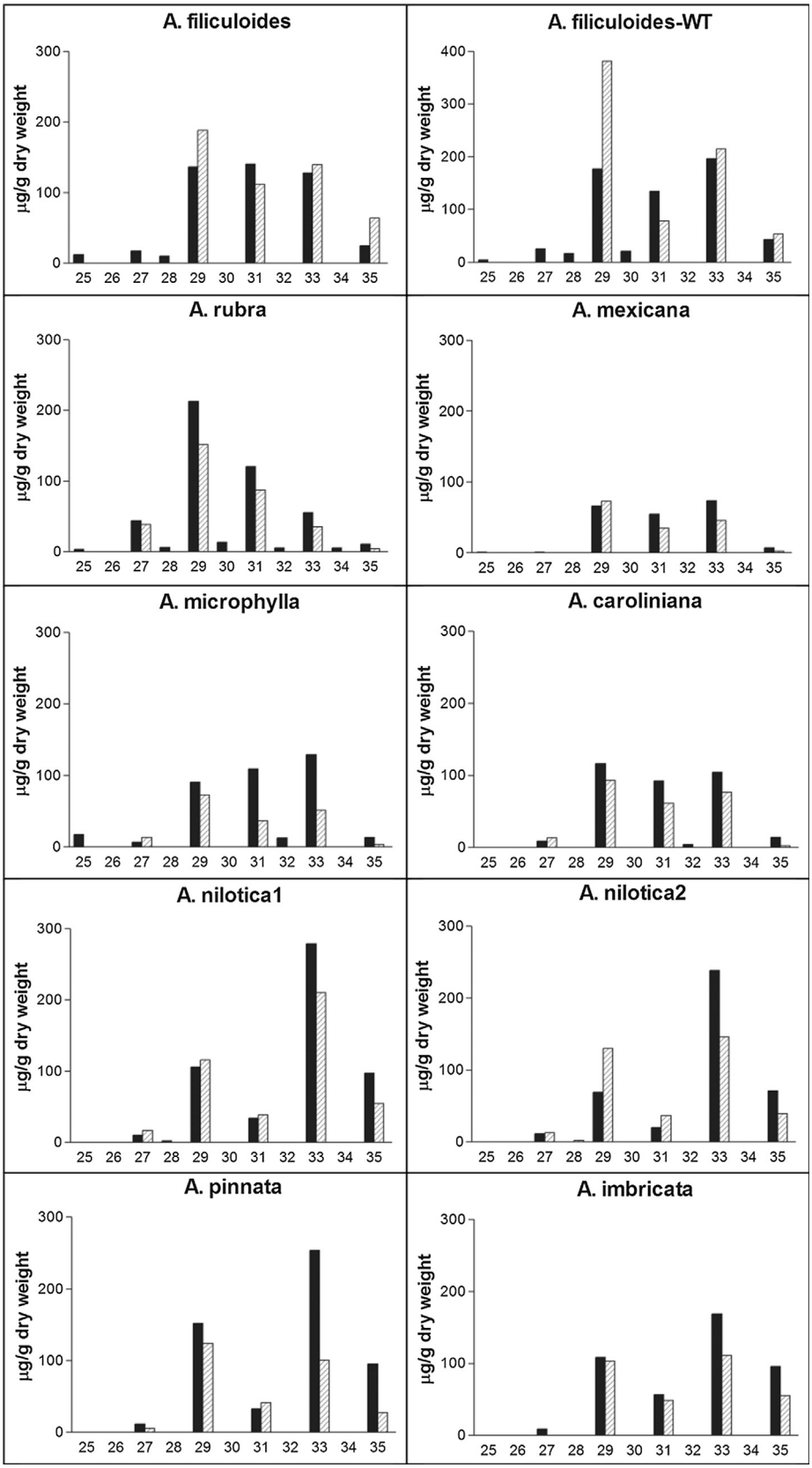

Fig. 3. Chain length distribution of the ω20-alkanols in total lipid extracts (black bars) and saponified extracts (grey scaled bars) from different *Azolla* species.

A. mexicana contained the least free and ester-bound 1,ω20-diols, while both *A. nilotica* species had the highest contents. Generally, C_{32}, C_{34} and C_{36} 1,ω20-diols are equally abundant with only minimal, if any, contributions of C_{30}. Only in the two *A. nilotica* varieties and *A. rubra* did the relative abundance of the diols decrease in the order $C_{32} > C_{34} > C_{36}$. Upon saponification, C_{32} was

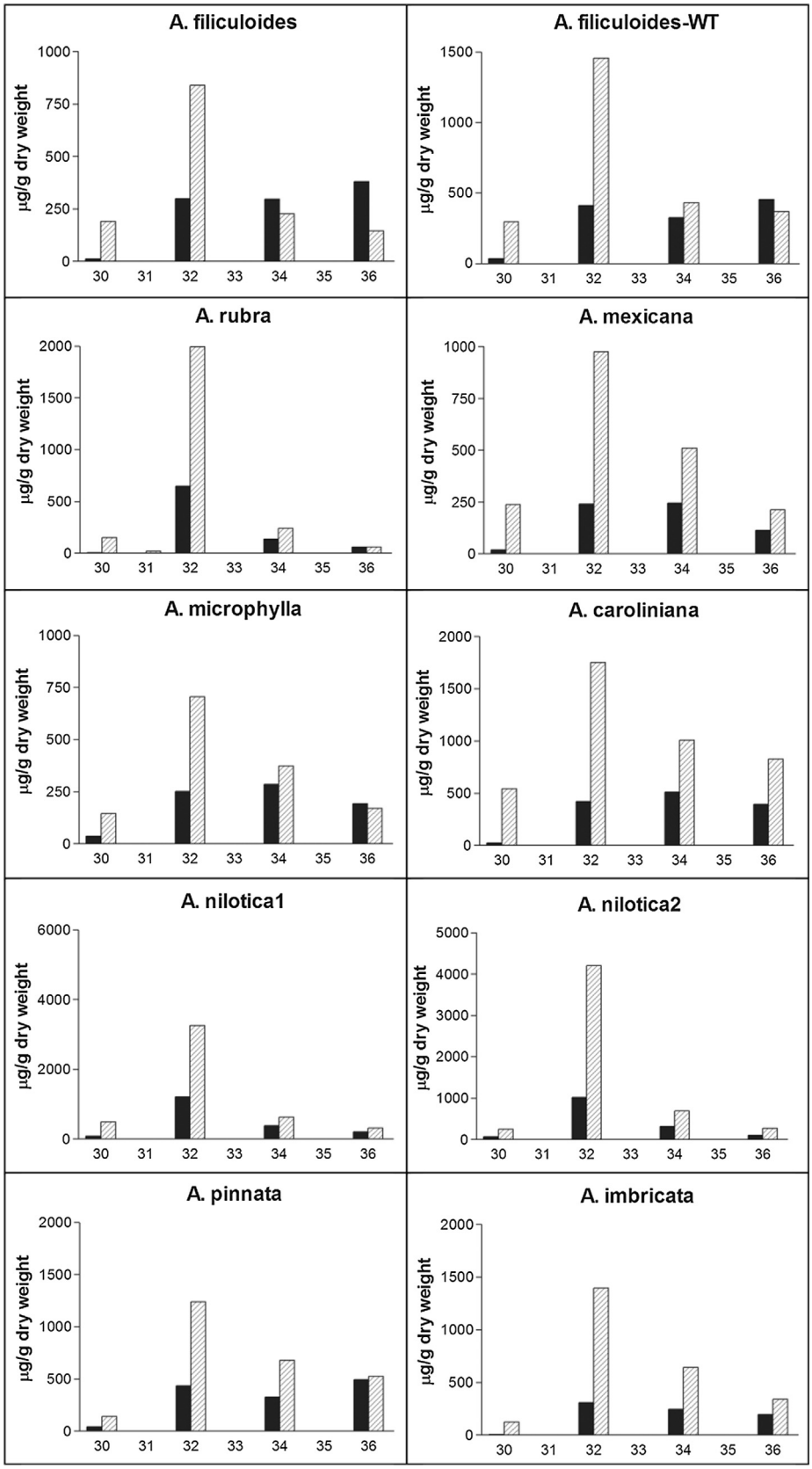

Fig. 4. Chain length distribution of the 1,ω20-diols in total lipid extracts (black bars) and saponified extracts (grey scaled bars) from different *Azolla* species.

the dominant 1,ω20-diol for all species. Esterified C_{30} 1,ω20-diols are released upon saponification, particularly from species belonging to the *Azolla* section, and to a much lesser extent from those belonging to the *Rhizosperma* section.

Even-numbered ω20-hydroxy fatty acids ranging from C_{26} to C_{36} were found in TLEs and SEs from all *Azolla* species. Free ω20-hydroxy fatty acids are dominated by either C_{32} (*A. rubra*), C_{34} (*A. mexicana, A. microphylla, A. caroliniana,* and *A. imbricata*)

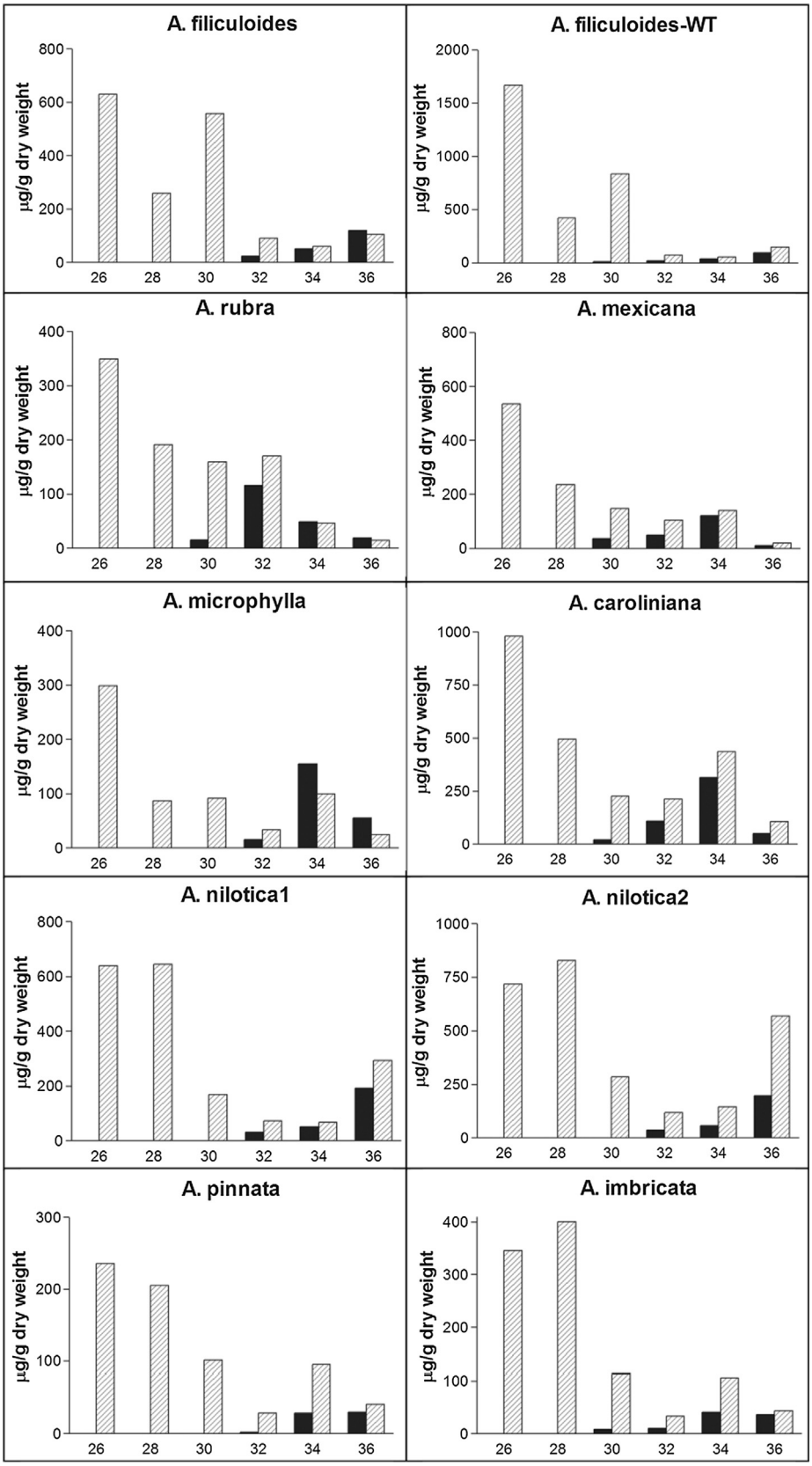

Fig. 5. Chain length distribution of the ω20-hydroxy fatty acids within the various *Azolla* species in total lipid extracts (black bars) and saponified extracts (grey scaled bars).

or C_{36} (*A. filiculoides, A. pinnata* and *A. nilotica* 1 and 2). In contrast, the ester-bound ω20-hydroxy fatty acids are of shorter chain length, being dominated by either C_{26} or C_{28} compounds (Fig. 5). Overall, ester-bound C_{26} ω20-hydroxy fatty acids are roughly twice as abundant as C_{28} in species of the *Azolla* section, while they are about equally abundant in species of the *Rhizosperma* section.

The ω20-hydroxy compounds identified in *Azolla* are in agreement with Speelman et al. (2009a) and Brouwer et al. (2016) for *A. filiculoides*, Atwood et al. (2014) for *A. microphylla*, and Mao et al. (2017) for *A. imbricata*. We found no difference in chain lengths (C_{26}–C_{36}) between the various hydroxy compounds of *A. microphylla* and all other *Azolla* species in contrast to a previous report (Atwood et al., 2014).

3.3. ω9,ω10-Dihydroxy compounds in Azolla species

Apart from the aforementioned mid-chain monohydroxy lipids, a second series of related compounds bearing two adjacent secondary hydroxy groups have been identified in *Azolla*; they occur predominantly as C_{29} compounds but exist as diols, triols and dihydroxy fatty acids as do the ω20-hydroxy compounds (Speelman et al., 2009a). Their mid-chain functional groups are at ω9 and ω10, and therefore, the number of carbons between the mid-chain hydroxy groups to the functional group at the 1 position (for the triols and dihydroxy fatty acids) is always the same, i.e. 19 and 20 carbon atoms, whereas for the ω20-hydroxy compound this depends on the chain length of the 1,ω20-diols and ω20-hydroxy fatty acids, respectively.

In the present study, most *Azolla* species contained ω9,ω10-diols almost exclusively as C_{29} (i.e., 20,21-diol) compounds, but C_{25} and C_{27} were also identified in much smaller concentrations. After hydrolysis, hardly any additional ω9,ω10-diols were released indicating that they are not generally esterified (Fig. 2). In contrast, the 1,ω9,ω10-triol and ω9,ω10-dihydroxy fatty acid which also occur solely as C_{29} compounds are mainly esterified; in particular, the dihydroxy fatty acid is hardly present as a free acid.

3.4. Esterified ω20-hydroxy and ω9,ω10-dihydroxy compounds in extant Azolla

C_{30} and C_{32} 1,ω20-diols and C_{29} 1,ω9,ω10-triol linked to a C_{16} fatty acid were identified using GC–MS (Speelman et al., 2009a, Brouwer et al., 2016). Palmitate esters may not be the sole wax esters in *Azolla,* however, wax esters of larger size are not generally amenable by GC–MS. Instead of direct detection, the extent to which the (di)hydroxy compounds are esterified is generally estimated based on the difference in concentrations between SEs and TLEs. *Azolla* ω20-alkanol and ω9,ω10-diol concentrations did not generally increase in the SEs and, therefore, were not part of the ester pool (Fig. 2); this is consistent with the absence of a functional group at the 1 position required for linear wax esters. In contrast, the amounts of ω20-diols generally increased in SEs compared to TLEs; they increased from 2 times (*A. pinnata* and *A. filiculoides*) to almost 4 times (*A. caroliniana*, *A. imbricata* and *A. nilotica* 2), whereas the concentration of ω20-hydroxy fatty acids on average increased 8-fold from TLEs to SEs. The 1,ω9,ω10-triols were mostly present as esters: upon saponification their abundances increased from 4 times (*A. pinnata*) to 22 times (*A. nilotica 2*), whereas the ω9,ω10-dihydroxy fatty acids increased from 11 (*A. pinnata* and *A. nilotica 1*) up to 61 fold (*A. filiculoides*). A large proportion of hydroxy compounds with a functional group at the 1 position, we conclude, was present as esters; this is analogous to 1-alkanols esterified to fatty acids in the cuticular waxes of seed plants (e.g., Kunst et al., 2008).

Although palmitate esters of 1,ω20-diols and 1,ω9,ω10-triols were identified in TLEs, esters composed of ω20-hydroxy fatty acids or ω9,ω10-dihydroxy fatty acids were not detected. Since no primary alkanols were identified upon saponification, the (di) hydroxy fatty acids are not generally esterified to 1-alkanols as in seed plants. Rather these fatty acids may combine with 1,ω20-diols and 1,ω9,ω10-triols to form high molecular weight esters that are not GC-amendable.

3.5. The chain length distribution of ω20-hydroxy compounds discriminates fern species from the different Azolla sections

Based on the compositions of the various ω20-hydroxy compounds we found the largest differences between the *Azolla* species in chain length distribution for the ω20-alkanols and the saponified ω20-hydroxy fatty acids. Cluster analysis was performed to determine whether these differences could be statistically related to membership of each variety to the *Azolla* and *Rhizosperma* sections. When considering the normalized concentrations of ω20-alkanols in TLEs, varieties belonging to the section *Azolla* were clustered together vs varieties belonging to the section *Rhizosperma*. A similar clustering was obtained for ω20-alkanols concentrations in SEs. An ANOVA test showed that for both TLEs and SEs the concentrations of the C_{29}, C_{31}, C_{33}, C_{35} ω20-alkanols differed significantly ($P < 0.05$) between the two clusters, whereas C_{31} and C_{35} ω20-alkanols were most discriminant based on their F-values (Supplementary Table S1).

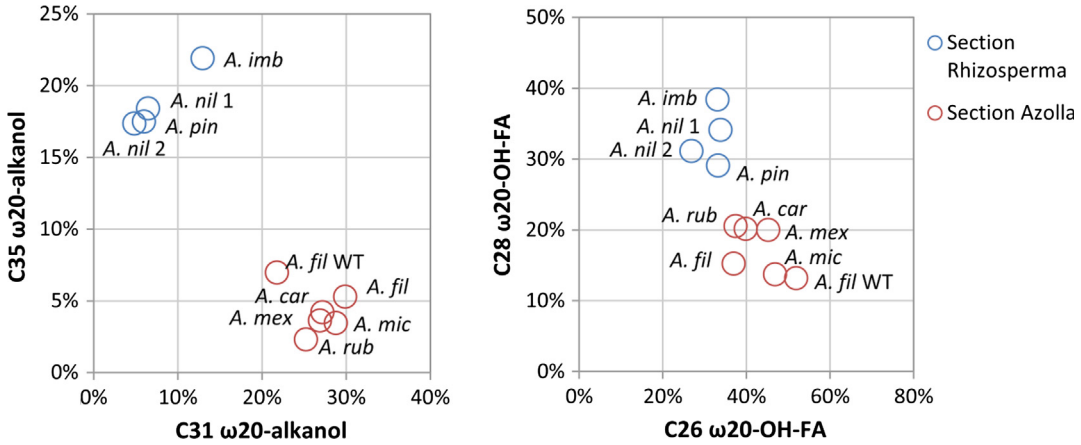

Fig. 6. Plots relating the C_{31}–C_{35} ω20-alkanol ratio in total lipid extracts (A) and the C_{26}–C_{28} ω20-hydroxy fatty acid ratio in saponified extracts (B) for all *Azolla* species and varieties analysed.

Clustering based on normalized concentrations of ω20-hydroxy fatty acids in SEs grouped members of the two sections together, with the exception of *A. caroliniana*, which was placed together with the species belonging to the *Rhizosperma* section. An ANOVA indicated significant differences ($P < 0.05$) between clusters for concentrations of C_{26}, C_{28} and C_{30} ω20-hydroxy fatty acids, whereas concentrations of C_{26} and C_{28} ω20-hydroxy fatty acids discriminated best between groups based on their F-value (Supplementary Table S1). The placement of *A. caroliniana* together with ferns of the *Rhizosperma* section is caused by the concentration of the C_{30} ω20-hydroxy fatty acid deviating from that in other species of the *Azolla* section. However, when performing the cluster analysis using solely the concentrations of the C_{26} and C_{28} ω20-hydroxy fatty acids *A. caroliniana* is placed among ferns of the *Azolla* section with high confidence (Supplementary Table S1).

Hence, based on the ANOVA results we define two ratios that can be used to discriminate between members of the *Azolla* and *Rhizosperma* sections: C_{31}/C_{35} ω20-alkanol and C_{26}/C_{28} ω20-hydroxy fatty acid. The C_{31}/C_{35} ω20-alkanol ratio is best derived from TLEs to minimize the overshadowing of these compounds by other components in SEs, whereas determination of the C_{26}/C_{28} ω20-hydroxy fatty acid ratio requires saponification of TLEs. Fig. 6 depicts these ratios for all varieties analysed. The average ratio of C_{31}/C_{35} ω20-alkanol in TLEs is 7.04 ± 2.66 for the species of the section *Azolla* vs 0.39 ± 0.14 for species of the *Rhizosperma* section. The C_{26}/C_{28} ω20-hydroxy fatty acid ratio is on average 2.65 ± 0.85 for members of section *Azolla* and 0.97 ± 0.13 for members of section *Rhizosperma*. A two-way homoscedastic *t*-test confirmed that both ratios differ significantly between the two sections (P-value = 0.001 and 0.005, respectively). Hence, *Azolla* species belonging to section *Azolla* are characterized by significantly higher concentrations of C_{31} ω20-alkanols compared to C_{35}, and roughly twice as much C_{26} ω20-hydroxy fatty acid than C_{28}, which distinguishes them from members of section *Rhizosperma* that contain more C_{35} than C_{31} ω20-alkanol and equal amounts of C_{26} and C_{28} ω20-hydroxy fatty acids.

3.6. Are the long chain ω20-hydroxy and ω9,ω10-dihydroxy compounds unique to Azolla?

To investigate whether the occurrence of these compounds is truly unique to species of the genus *Azolla* we investigated a set of representative species of closely related genera for the occurrence of ω20-hydroxy and/or ω9,ω10-hydroxy compounds, as well as two floating aquatic monocots, *Lemna trisulca* and *Pistia stratiotes*, that thrive in similar habitats. The genetically unrelated floating plants, *L. trisulca* and *P. stratiotes*, contained neither ω20-hydroxy nor ω9,ω10-hydroxy compounds (Fig. 7), but common *n*-alkanes, *n*-alkanols and fatty acids instead. *P. stratiotes* contains a 1,15 keto-ol (C_{32}). Species belonging to the Marsileaceae family of aquatic ferns, *M. quadrifolia*, *M. hirsuta*, *Pilularia globulifera*, and *Regnellidium diphyllum*, contained non-cyclic lipids such as *n*-alkanes, *n*-alkanols and fatty acids, but no ω20-hydroxy and ω9,ω10-dihydroxy compounds were identified (Fig. 7).

In contrast, ω20-hydroxy and ω9,ω10-dihydroxy compounds were detected in all six *Salvinia* species analysed here, indicating that the biosynthesis of the ω20-hydroxy and ω9,ω10-dihydroxy components must have evolved early in Salviniaceae evolution (>89 Ma). Hence, ω20-hydroxy and ω9,ω10-dihydroxy

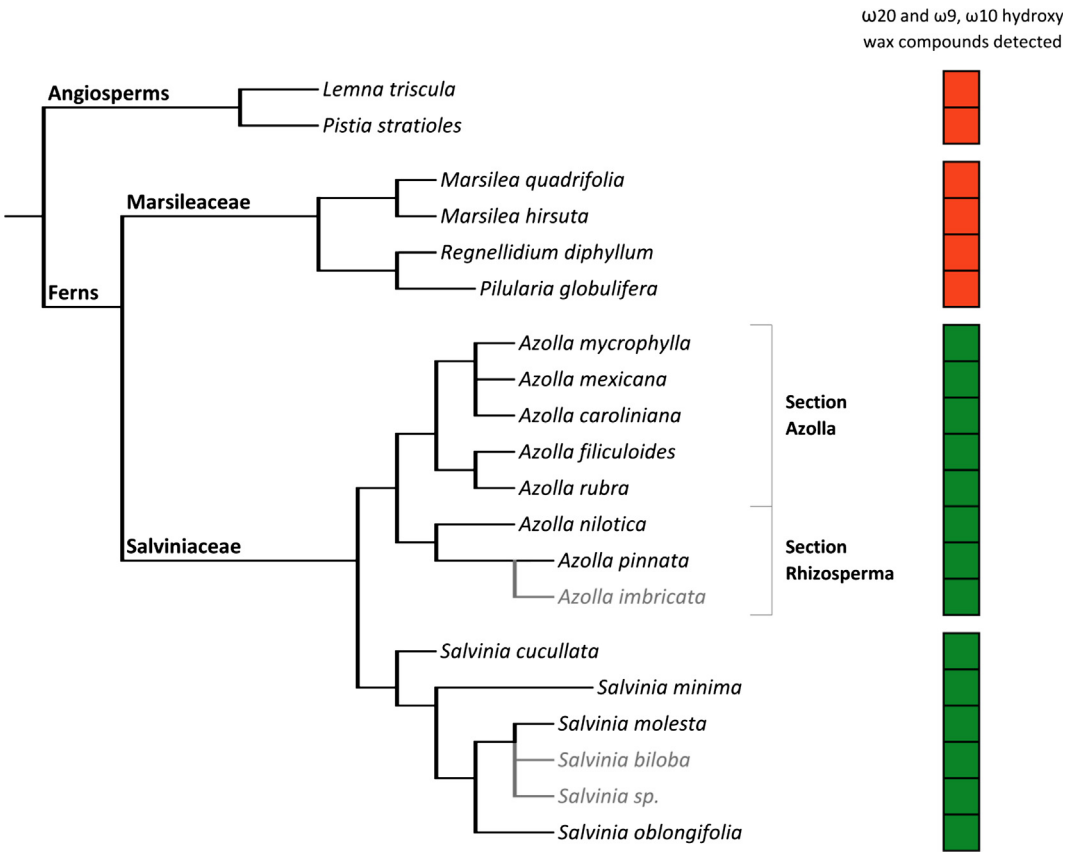

Fig. 7. Cladogram of species analysed in this paper (after Nagalingum et al., 2008) and occurrence (green: present, red: absent) of ω20-hydroxy and the ω9,ω10-dihydroxy compounds.

compounds, we conclude, are not unique to the *Azolla* genus, but rather unique to ferns of the Salviniaceae family (Fig. 7).

3.7. Distribution of ω20-hydroxy and ω9,ω10-dihydroxy compounds in Salvinia

The proportion and chain length distribution of the individual alkanols, diols and hydroxy fatty acids with the ω20 functionality are noticeably different in *Salvinia*, compared to all *Azolla* species (Fig. 8). In *Salvinia* C_{28} and C_{30} ω20-hydroxy fatty acids, C_{31} 1, ω20-diol, C_{29} 1,ω9,ω10-triol and C_{29} ω9,ω10-dihydroxy fatty acid predominate whereas other analogues occur in much smaller concentrations (Supplementary Fig. S1–4). The latter comprise odd numbered ω20-alkanols (C_{27}–C_{35}), 1,ω20-diols (C_{30}–C_{32}), even numbered ω20-hydroxy fatty acids (C_{26}–C_{32}, as well as traces of C_{31}), and the three ω9,ω10-dihydroxy compounds (C_{29}). *Salvinia* has, therefore, a less diverse wax composition than *Azolla*. Compounds found in *Azolla* but not *Salvinia* were: C_{25} and even numbered ω20-alkanols, C_{34} and C_{36} 1,ω20-diols and C_{34} and C_{36} ω20-hydroxy fatty acids. Conversely, *Salvinia* contains both C_{31} 1, ω20-diol and C_{31} ω20-hydroxy fatty acid that were absent from *Azolla*. Hence, C_{31} 1,ω20-diol and ω20-hydroxy fatty acid are diagnostic for *Salvinia*, whereas particularly C_{34} and C_{36} 1,ω20-diols and ω20-hydroxy fatty acids are unique for *Azolla*.

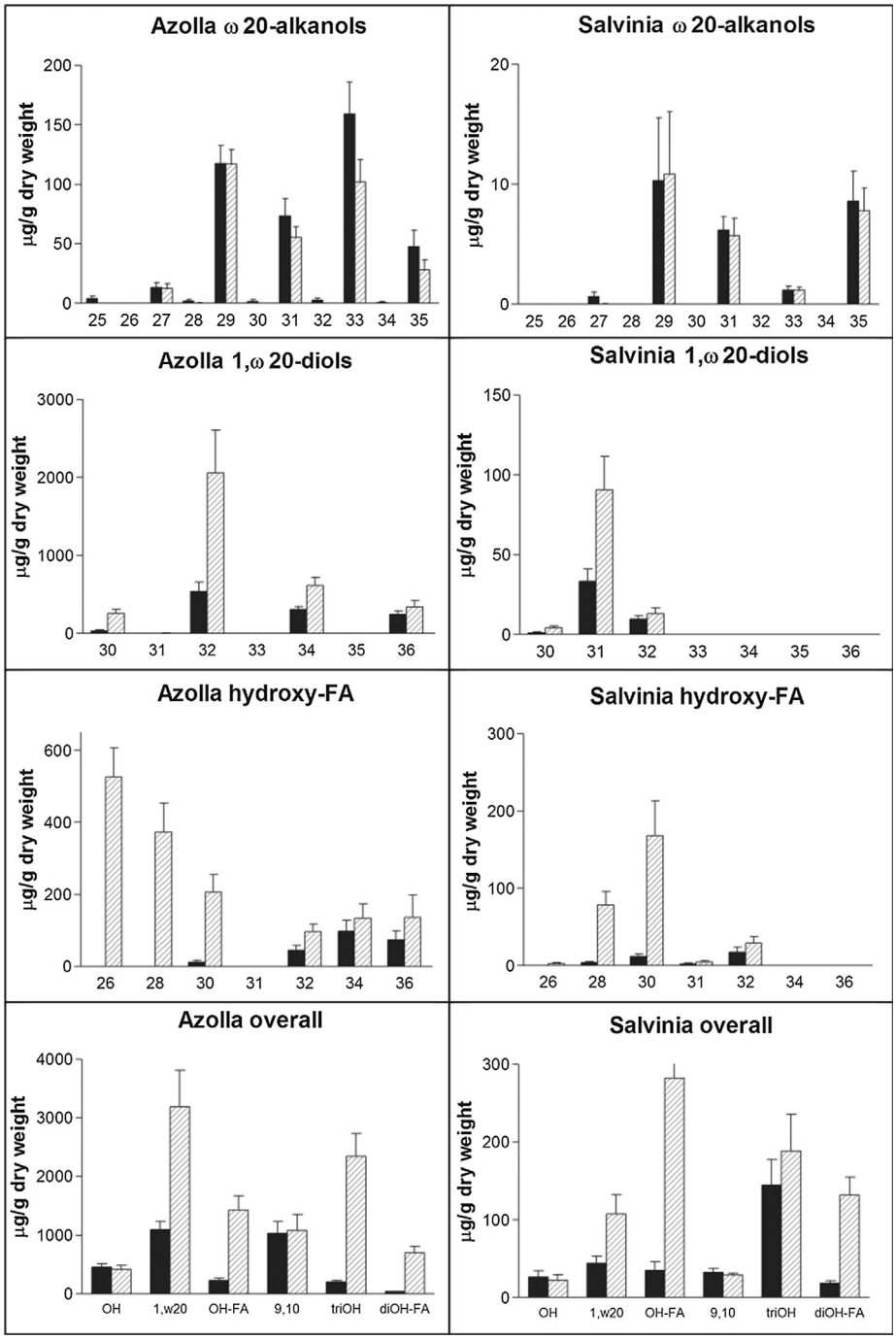

Fig. 8. Composition of ω20-hydroxy and ω9,ω10-dihydroxy compound groups in *Azolla* and *Salvinia* in total lipid extracts (black bars) and saponified extracts (grey scaled bars). Abbreviations are described in the Fig. 2 legend. Data points represent averages from all species combined and their standard deviations.

In addition to mid-chain hydroxy functionalized compounds, traces of (C_{32}) 1,ω20-keto-ol were also identified in *Salvinia* species (mass spectrum *m*/*z* 130, 143, 300, 382, 537 (M^+–15)) (Versteegh et al., 1997; Zhang et al., 2011)).

3.8. Biosynthesis of ω20-hydroxy and ω9,ω10-dihydroxy compounds

Leaves of *A. filiculoides* contain ω20-hydroxy and ω9,ω10-dihydroxy lipids, whereas the roots, mega- and microsporocarps do not. The only exception is a very small trace of C_{29} 1,ω9,ω10-triol upon hydrolysis of the TLE of the roots. The presence of the ω20-hydroxy and ω9,ω10-dihydroxy lipids in leaves and their virtual absence from the other tissues suggests their localization in the cuticular waxes similar to seed plants (Speelman et al., 2009a). The same holds for ferns from the genus *Salvinia*: leaves from *S. oblongifolia* were rich in ω20-hydroxy and ω9,ω10-dihydroxy lipids; in contrast, roots of *S. oblongifolia* only contained a trace amount of C_{30} hydroxy fatty acid upon saponification of the TLE. *S. oblongifolia* does not contain cyanobacteria in its leaves in contrast to *A. filiculoides* which rules out the possibility that the lipids are synthesized by cyanobacteria.

The wax composition in Salviniaceae clearly differs from that in other land plants by the consistent introduction of ω20- or ω9- and ω10-hydroxy groups. In seed plants wax biosynthesis starts with the Fatty Acid Elongation (FAE) pathway, leading to the synthesis of very-long-chain fatty acyl-CoAs, which consist of co-enzyme A linked to the very-long-chain fatty acid side chain (Bernard and Joubès, 2013). These subsequently serve as substrate for different enzymes that catalyse the release of fatty acids, sometimes referred to as very long chain fatty acids (VLCFAs), the conversion into *n*-alkanes and the reduction into 1-alkanols. Part of the 1-alkanols are esterified thus forming wax esters. Mid-chain hydroxy compounds are generally believed to be formed by the hydroxylation of *n*-alkanes. However, as previously noted by Speelman et al. (2009a) the fixed position of a hydroxy group at ω20 indicates that compounds are synthesized by chain elongation of a parent secondary alcohol, rather than by hydroxylation of *n*-alkanes.

Since VLCFA derivatives were found as ω20-hydroxy fatty acids, ω20-alkanols and 1,ω20-diols of differing chain lengths, they are more likely to be derived from a common ω20-hydroxy precursor, possibly C_{26}, elongated by the FAE complex(es) (Fig. 9). The even-over-odd predominance and chain length distribution of the 1,ω20-diols and ω20-hydroxy fatty acids reflect short chain fatty acid biosynthesis from malonyl-CoA in chloroplasts and is similar to that of 1-alkanols and fatty acids in land plants. The C_{31} 1,ω20-diol in *Salvinia* constitutes an interesting exception to this. The strong odd-over-even predominance of ω20-alkanols in ferns from the Salviniaceae suggests synthesis by decarboxylation common to *n*-alkane biosynthesis in land-plant waxes (Walton, 1990). ω20-Hydroxy lipids in species of the Salviniaceae, however, generally have a longer chain length than those of other land plants (Walton, 1990). The latter is true particularly for the 1,ω20-diols in *Azolla* that range from C_{30} to C_{36}, while in other plants 1-alkanols are mainly within the range of C_{22}–C_{28} and only limited amounts of C_{30}–C_{34} moieties have been identified (Kolattukudy, 1980; Walton, 1990).

Additionally, genes encoding enzymes homologous to those catalysing synthesis VLCFAs in seed plants have been detected in *Azolla* (Brouwer et al., 2014). Annotation of the *A. filiculoides* transcriptome using the Kyoto Encyclopedia of Genes and Genomes (KEGG) revealed expression of all the enzymes in the FAE pathway as well as those required to convert ω20-hydroxy acyl CoAs into ω20-alkanols, 1,ω20-diols and ω20-hydroxy fatty acids (Brouwer et al., 2014). Enzymes required for esterification of 1,ω20-diols with palmitic acid were missing from this analysis, but their absence may be related to limitations in transcriptome assembly and annotation. The enzyme(s) catalysing ω20-hydroxylation remain(s) unknown, but may belong to the cytochrome P450 superfamily. In order to identify and prove the function of such mid-chain hydroxylase, sequences from transcripts of tissues

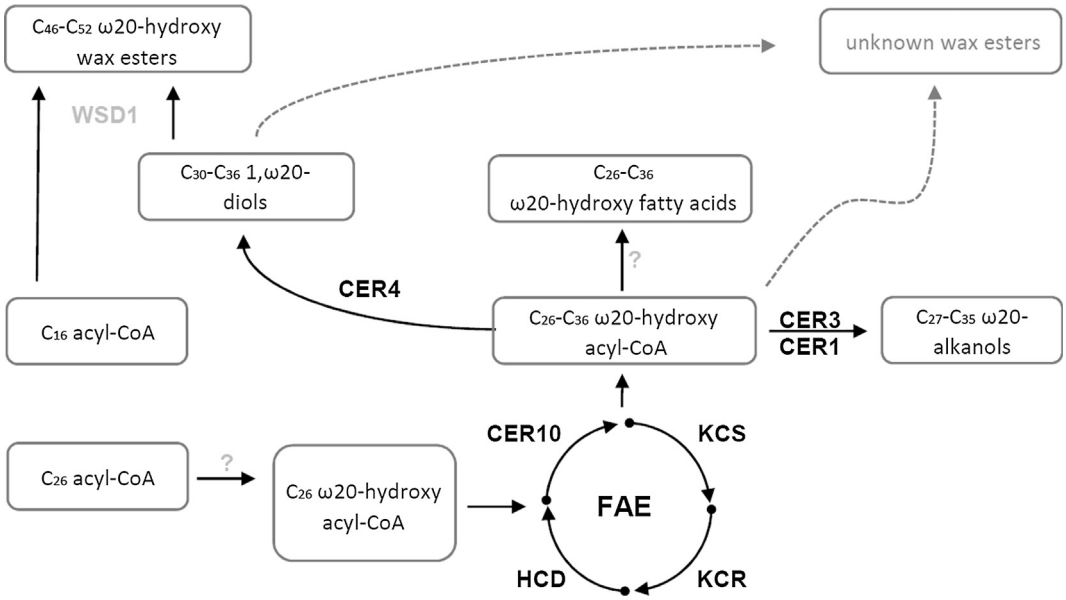

Fig. 9. Suggested biosynthetic pathways of ω20-hydroxy and ω9,ω10-dihydroxy VLCFA derivatives in *Salviniaceae* based on known enzymes of plant wax biosynthesis pathways. In higher plants, CER10, HCD, KCR and KCS are enzymes of the Fatty Acid Elongation (FAE) complex(es) which will elongate the C_{26} ω20-hydroxy acyl-CoA with acyl-CoA up to C_{36}, while also releasing intermediates C_{28}–C_{34}. Likely, an Eceriferum3 (CER3) enzyme reduces and a CER1 enzyme subsequently decarbonylates the even numbered VLCFA thioesters to odd numbered C_{27}–C_{35} ω20-alkanols. Release of the thioesters as free fatty acids may be performed by an, as yet unknown, thiolase. A Fatty Acid Reductase enzyme (CER4) likely converts the even numbered FA thioesters into even numbered C_{26}–C_{36} 1, ω20-diols, which likely become esterified by a Wax Synthase/Diacylglycerol acyl transferase 1 (WSD1) (Bernard and Joubès, 2013). When depicted in black, similar enzymes were identified in the *Azolla* transcriptome by annotation using the Kyoto Encyclopedia of Genes and Genomes (KEGG). 'Unknown wax esters' refers to the combination of 1,ω20-diols and ω20-hydroxy fatty acids that could not be identified by GC–MS analysis, whereas elevated concentrations were observed after saponification of total extracts.

expressing the enzyme may be compared to those that do not express it; for example, by comparing sporophyte with sporocarp or root transcripts. Alternatively, sporophyte transcripts from ferns of the Salviniaceae could be compared with those from a fern of the Marsileaceae.

In *Azolla*, linear lipids of C_{26} and longer chain lengths contained a ω20-hydroxy group. Small amounts of C_{26} fatty acids were also detected. Hydroxylation, therefore, likely occurred on the C_{26} acyl-CoA or on shorter acyl chains which were not released by FAE. In *Salvinia* C_{26} ω20-hydroxy compounds were hardly detected compared to *Azolla*. Instead, the C_{28} fatty acid was identified, suggesting that in *Salvinia* the hydroxylating enzyme prefers the C_{28} acyl-CoA as substrate or the FAE releases longer chains. The differences in distribution of ω20-hydroxy compounds observed between *Azolla* sections and *Azolla* and *Salvinia* species likely also reflect preferred substrate chain-length of the enzymes converting the pool of ω20-acyl CoAs into ω20-alkanols, 1,ω20-diols and ω20-hydroxy fatty acids. Hence, future sequencing information may be used to link chain-length preferences with specific mutations in the key enzymes. Combined with geochemical analysis of geological records, this will allow dating the evolution of the biochemical pathways in these species as well as the emergence of their common ancestors.

In contrast to the biosynthesis of ω20-hydroxy compounds, the biosynthesis of the odd numbered ω9,ω10-dihydroxy compounds (diols, triols and dihydroxy fatty acids) dominated by C_{29} is less straightforward. Mao et al. (2017) propose that starting from the C_{29}-dihydroxy fatty acid the triol is formed after acyl reduction, hydrogenation and dehydration, and then again hydrogenation and dehydration would form the ω9,ω10-diol. The precursors and origin of the C_{29} dihydroxy fatty acid, however, remains unclear. Speelman et al. (2009a) suggested formation of C_{29} ω9, ω10-hydroxy compounds from decarboxylation of corresponding diacids, but no evidence for this biosynthetic pathway has been found. Furthermore, the *Salvinia* marker C_{31} 1,ω20-diol could be a product of the corresponding ω20-hydroxy fatty acid, but production of the C_{30} ω20-alkanol by decarboxylation is not observed. Hence, although most ω20-hydroxy compounds fit the generally accepted model for wax biosynthesis in seed plants, the formation of ω9,ω10-dihydroxy compounds in Salviniaceae species as well as the sudden odd-predominance of the C_{31} 1,ω20-diol in *Salvinia* need further investigation.

3.9. ω20-Hydroxy and ω9,ω10-dihydroxy compounds in the geological record

The similar ranges of ω20-alkanols in the Arctic Eocene sediments and in extant *Azolla* species, suggests that the Eocene ω20-alkanols could be a direct reflection of the original signal produced by species of Eocene Arctic *Azolla*, and confirm the relative resistance of the ω20-hydroxy compounds to degradation.

Speelman et al. (2009a) identified most of the ω20-hydroxy and ω9,ω10-dihydroxy compounds in Eocene sediments and concluded that they preserved well in the geological record. 1,ω20-Diols as well as both C_{29} ω9,ω10-dihydroxy lipids (9,10-diol and 1,20,21-triol) have also been described in recent lake sediments containing *A. microphylla* fossil remains (Zhang et al., 2011). C_{29} ω9,ω10-dihydroxy compounds were less abundant than the ω20-hydroxy components in the lake sediments compared to extant *Azolla* implying a lesser stability of the ω9,ω10-dihydroxy lipids also observed by Speelman et al. (2009a). Both studies indicate that the biomarkers containing an acid functionality are more prone to

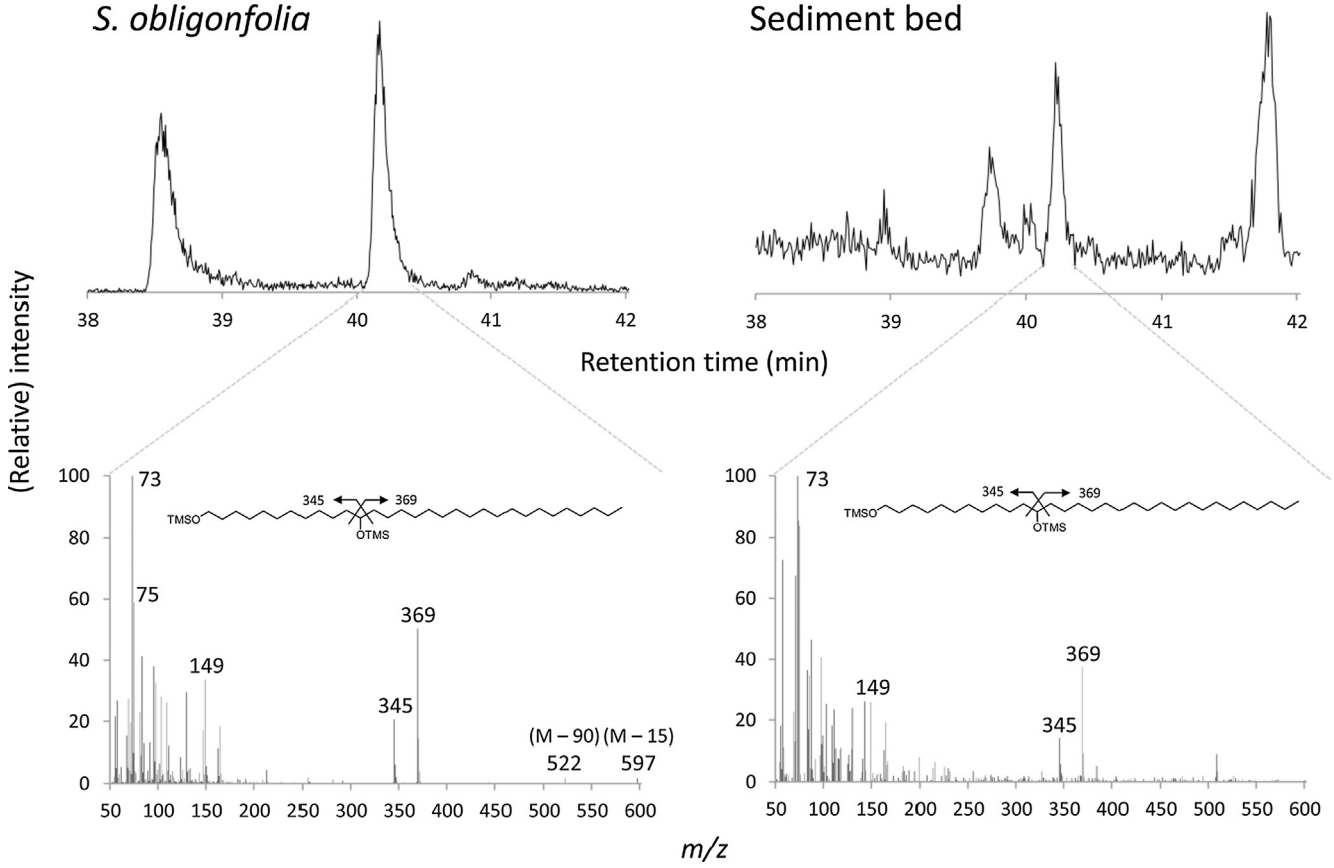

Fig. 10. Partial gas chromatograms of saponified extracts of *S. oblongifolia* leaves and sediment bed of the Kreftenheye Formation showing the peaks and mass spectra of C_{31} 1, ω20-diol.

degradation than those without, consistent with the relative reactivity of lipids in Black Sea sediments of fatty acids > alkyl diols (Sun and Wakeham, 1994).

Although not recognized in the Eocene sediments (Speelman et al., 2009a), C_{27} and C_{28} 9,10-diols have been found in recent lake sediments (Zhang et al., 2011). It has been suggested that the C_{27} and C_{28} 9,10-diols may, therefore, result from either diagenesis or a biosynthetic response of *Azolla* to specific environmental conditions (cf., Zhang et al., 2011). Since we have found all compounds in extant *A. filiculoides*, diagenesis does not need to be invoked. Additionally, the occurrence of these dihydroxy lipids in various *Azolla* species from different sample sites may dismiss a specific environmental effect on biosynthesis.

The distribution of (free) 1,ω20-diols in Eocene sediments was almost identical to that of extant *A. filiculoides* investigated by Speelman et al. (2009a). However, from our research it appears that extant *Azolla* species of the *Azolla* section and *Rhizosperma* section produce clearly different distributions of particularly ω20-alkanols and ω20-hydroxy fatty acids. These differences were unlikely present during the Eocene, since the *Azolla* and *Rhizosperma* sections likely diverged during that period and not before (Metzgar et al., 2007). Analysis of the ω20-alkanol distributions in more recent sediments may permit dating emergence of common ancestors of the ferns in either section of the *Azolla* genus.

Given the identification of ω20-hydroxy and ω9,ω10-dihydroxy compounds in extant *Salvinia* species we wondered whether these could also be observed in sediments known to contain *Salvinia* fossils. Sediments of the *Salvinia* bed (Kreftenheye Formation) from Raalte, The Netherlands, have been previously studied for plant remains and were shown to contain abundant amounts of *Salvinia* spores (Van der Ham et al., 2008). The lipid composition of a section at 10–17 m was analysed and proved devoid of the ω20-hydroxy and ω9,ω10-dihydroxy compounds common in *Azolla*, but trace amounts of the C_{31} 1,ω20-diol characteristic of *Salvinia* confirmed its presence in Eemian sediments (100 kA) and validates the C_{31} 1,ω20-diol molecular marker (Fig. 10). The C_{31} 1,ω20-diol has not been identified in Eocene sediments, nor in recent sediments with clear evidence of *Azolla* inputs (Speelman et al., 2009a; Zhang et al., 2011). Given that the C_{31} 1,ω20-diol was the only detectable molecular remain in the *Salvinia* bed, this is consistent with its predominance in extant *Salvinia* species and illustrates its value as a biomarker distinguishing *Azolla* from *Salvinia*.

4. Conclusions

All fern species of the family Salviniaceae contain a series of long chain ω20-hydroxy and ω9,ω10-dihydroxy lipids, of which the majority is in esterified form, most likely in their leaf cuticular waxes. Diversity and concentrations were higher in ferns from the genus *Azolla,* with *Azolla* ferns containing 10 times more (di)hydroxy compounds than *Salvinia* ferns. In contrast, the closely related ferns from the family Marsileaceae and some non-related genera of other floating aquatic plants did not contain these lipids. ω20-Hydroxy and ω9,ω10-dihydroxy compounds, therefore, are characteristic of ferns of the Salviniaceae. Moreover, C_{34} and C_{36} 1,ω20-diols and ω20-hydroxy fatty acids appear to be unique to *Azolla* whereas C_{31} 1,ω20-diol and ω20-hydroxy fatty acids are typical of *Salvinia* species. Given their extraordinary stability, in particular that of the 1,ω20-diols, these compounds are thus valuable biomarkers of these species in sedimentary records. Finally, since the distribution of the ω20-alkanols and the ω20-hydroxy fatty acids is characteristic in ferns of the two sections of the *Azolla* genus, the ratios of C_{31}/C_{35} ω20-alkanol and of C_{26}/C_{28} ω20-hydroxy fatty acid may therefore be used to distinguish the source at the section level.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.orggeochem.2018.09.014.

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