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A phytochemical and morphological study of the liverwort *Plagiochila retrorsa* Gottsche, new to Europe

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SUMMARY

The Neotropical liverwort *Plagiochila retrorsa* Gottsche is conspecific with *P. sharpii* H.L.Blomq., known previously as a Southern Appalachian endemic, as well as *P. tricarinata* Carl and *P. permista* Spruce var. *subintegerrima* Herzog from Costa Rica. It has been discovered recently in the Azores and Madeira, new to Europe. Illustrations, SEM micrographs of spores and elaters, nuclear magnetic resonance (NMR) fingerprints and gas chromatography–mass spectrometry (GC–MS) data are presented, based on American and European material. Costa Rican material used for an earlier phytochemical study has now also been identified as *P. retrorsa*. Although NMR fingerprinting initiated the present discoveries, the NMR fingerprint of *P. retrorsa* is not unique and NMR fingerprinting alone is not sufficient to identify the species. *P. retrorsa* belongs to the 9,10-dihydrophenanthrene chemotype group and the two principal secondary metabolites are 3,5-dimethoxy-9,10-dihydrophenanthren-2-ol and methyl 4-hydroxy-4′-*O*-methyllunularate.

KEYWORDS: Plagiochilaceae, *Plagiochila retrorsa*, NMR spectroscopy, chemosystematics, chemotaxonomy, morphology, distribution.

INTRODUCTION

Identification of species in the large genus *Plagiochila* is notoriously difficult and has resulted in the description of numerous species. Revision of Neotropical *Plagiochilae* is confirming extensive synonymy (e.g. Heinrichs & Gradstein, 2000) and the value of a combined morphological and chemotaxonomical approach has been demonstrated (Heinrichs *et al.*, 2000). Here we present further results of complementary chemical and morphological studies, where an initial phytochemical observation has led to addition of the Neotropical liverwort *Plagiochila retrorsa* Gottsche to the European flora.

In 1996, Elena Reiner-Drehwald and Uwe Drehwald collected a *Plagiochila* on Madeira first identified, along with others, as *Plagiochila killarniensis* Pearson (= *P. bifaria* (Sw.) Lindenb.; Heinrichs, Grolle & Drehwald, 1998) because of the presence of a distinct vitta. Phytochemical investigation of the specimen using nuclear magnetic resonance (NMR) fingerprinting (Rycroft, 1996, 1998a; Rycroft, Cole & Lamont, 1999b) revealed that the dominant secondary metabolites differed from those of European populations of *P. bifaria* (Rycroft *et al.*, 1999a), including Madeiran ones (Rycroft, 1999), but resembled those of an unidentified *Plagiochila* from Costa Rica (Anton *et al.*, 1997). This observation prompted closer examination of the morphology, serendipitously within a month of having learnt (Bates, *in litt.*, 1998) of Mrs J.A. Paton's discovery in a collection of *P. bifaria* from Terceira (Azores; coll. Bates & Gabriel, 1994) of two fragmentary shoots that appeared (notes in packet, 1995) to be *Plagiochila sharpii* H.L.Blomq., a species known from the Southern Appalachian mountains of the U.S.A. (Blomquist, 1940; Schuster, 1980). It was noted that the chemically different Madeiran specimen and the unidentified Costa Rican specimen also resembled *P. sharpii*. These results led to a detailed study of *P. sharpii* and related taxa, including fieldwork in Costa Rica and Madeira, and several older names for *P. sharpii* were traced by examination of type specimens. In addition, NMR and gas chromatography–mass spectrometry (GC–MS) fingerprinting have been used to investigate the principal lipophilic secondary metabolites in specimens from North America, the Central American Neotropics and Macaronesian Europe, including two voucher specimens from the earlier chemical investigation (Anton *et al.*, 1997).

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Materials and Methods

Plant material

The phytochemical studies used the upper parts of stems selected from some of the specimens listed under *Representative specimens examined.* Care was taken to remove epiphyllous stems of other hepatics.

NMR fingerprinting

Extracts were prepared by triturating dried plant material with sufficient $CDCl_3$ to produce 0.6–0.7 mL of a filtered solution. NMR fingerprints (Rycroft, 1996, 1998a; Rycroft, Cole & Rong, 1998) were obtained by measuring 400 MHz ^{1}H NMR spectra of the solutions. The weight (mg) of liverwort extracted and the concentration (mM) of 3,5-dimethoxy-9,10-dihydrophenanthren-2-ol in the extracts (based on NMR integration and comparison with the residual $CHCl_3$ signal) was, using the numbering of Table 1, (i) 100, 3, (ii) 29, 1, (iii) 31, 1, (iv) 86, 2, (v) 25, 1, (vi) 27, 1, (vii) 98, 0.05, (viii) 57, 3, (ix) 152, 3, (x) 75, 5, (xi) 31, 3, (xii) 39, 1. Compounds in the extracts and acetylation derivatives were also characterized *in situ* using GC and GC–MS.

GC–MS

GC and GC–MS were performed generally as described previously (Rycroft *et al.*, 1998). The GC column was calibrated with a mixture of *n*-alkanes so that Kováts retention indices could be determined. Data for the acetylated extracts were compared with data for the untreated extracts in order to identify compounds containing hydroxyl groups that could be acetylated. Because of strong adsorption in the inlet port or on the column (especially for extracts (i), (v) and (vii), that were run on a well-used column), peaks from some of the polar components were weak or absent until the extract was acetylated.

Morphological Study

Plagiochila retrorsa Gottsche, Mexik. Leverm.: 67. 1863. Type: Mexico. Veracruz: Pico de Orizaba, *Müller s.n.* (lectotype, here designated, G 026415 [ster.; herbarium Gottsche in B destroyed, no type specimen in C in 1999]).
= *Plagiochila tricarinata* Carl, Ann. Bryol. 2 (suppl 2): 74. 1931 (descriptio extensa in Herzog, Hedwigia 72: 224. 1932), **syn. nov.** Type: Costa Rica. San José: Cerro de las Vueltas, 2700–3000 m, *Standley & Valerio 43582* (holotype, JE [c.per., male]).
= *Plagiochila permista* Spruce var. *subintegerrima* Herzog, Rev. Bryol. Lichénol. 11: 12. 1939, **syn. nov.** Type: Costa Rica. San José: Cerro de las Vueltas, 2700–3000 m, *Standley & Valerio* 43536 (holotype, JE [male]).
= *Plagiochila sharpii* H.L.Blomq., Bryologist 43: 90. 1940, **syn. nov.** Type: U.S.A. North Carolina: Jackson County, White Water River Falls, *Anderson 6636* (holotype, DUKE 61676 [male]); Tennessee: Sevier County, Greenbrier, 2700 ft, *Sharp 3882* (paratype, DUKE 61675 [male]).

The species is illustrated in Figs 1–3.

Plants small to large in size, *ca* (10-)20–120(-160) mm long and (1.2-)2.3–4.5(-5) μm wide, in dense mats, dull green to brownish or olive-green. Main *stems* dorsally and ventrally flattened, near base *ca* 140–290 × (220-)240–370(-390) μm and 10–18 × 12–19 cells in diameter, brown, towards apex often greenish, with differentiation into short creeping stoloniferous shoots that give rise to leafy aerial stems, dorsally moderately exposed, ventrally often completely covered with leaves, cortical cells in 2–3(-4) layers, moderately to distinctly thick-walled, in cross section *ca* 18–25 (-30) × 12–20 μm, medullary cells thin-walled to slightly thick-walled, *ca* (14-)22–35(-38) × 14–28 μm, trigones absent or minute and triangular; *rhizoids* absent on leafy shoots (rarely present on short sections of leafy dorsal stem side attached to substratum). Vegetative *branches* lacking to moderate in number, of the lateral intercalary type. *Leaves* imbricate, on weak shoots sometimes remote, when moist postically secund, towards stem base sometimes ± widely spreading, in dry state erect to erect appressed and somewhat inrolled (small phenotypes) or leaf position ± equal to moist state (large phenotypes). Leaves asymmetrically triangular ovate to triangular with rounded to truncate apex, dorsally moderately to long decurrent, often running downwards to dorsal base of opposite leaf, ventrally hardly to moderately decurrent, *ca* (0.8-)1.1–2.0(-2.2) mm wide and (0.7-)1.3–2.3(-2.5) mm long, *ca* 0.9–1.6(-1.7) times as long as wide, dorsal margin moderately to distinctly recurved, in weak forms sometimes plane, ventral margin plane or recurved near base, mature stem leaves with (5-)13–38(-42) marginal teeth of which (2-)3–5 occur on apex, (0-)1–7(-8) on upper half of dorsal margin (rarely 1–2 teeth present on lower half of dorsal margin) and *ca* 3–33 on upper 5/6 of ventral margin; teeth straight or curved, triangular to elongate triangular, pointing in various directions, 1–4(-5) cells broad at base and (1-)2–5(-7) cells long, apical ones sometimes slightly coarser. *Cells* in upper leaf half slightly broader than long to elongate, *ca* 15–30(-35) × 14–23 μm and 0.7–1.9(-2.1) times as long as wide, at leaf base a rather short, broad vitta present, vitta cells *ca* (38-)45–75 (-83) × 17–25 μm and (1.6-)2–4.4(-4.9) times as long as wide; *trigones* small to large in size, triangular to nodulose, occasionally subconfluent, intermediate thickenings occasionally present on long walls of distinctly elongate cells of upper leaf half, the long walls of vitta cells often with strong, bulging to cone-like intermediate thickenings; *cell walls* thin except in 1–3 marginal cell rows with moderately to strongly thickened walls forming an indistinct yellowish border; *cuticle* smooth. *Oil* bodies homogeneous,

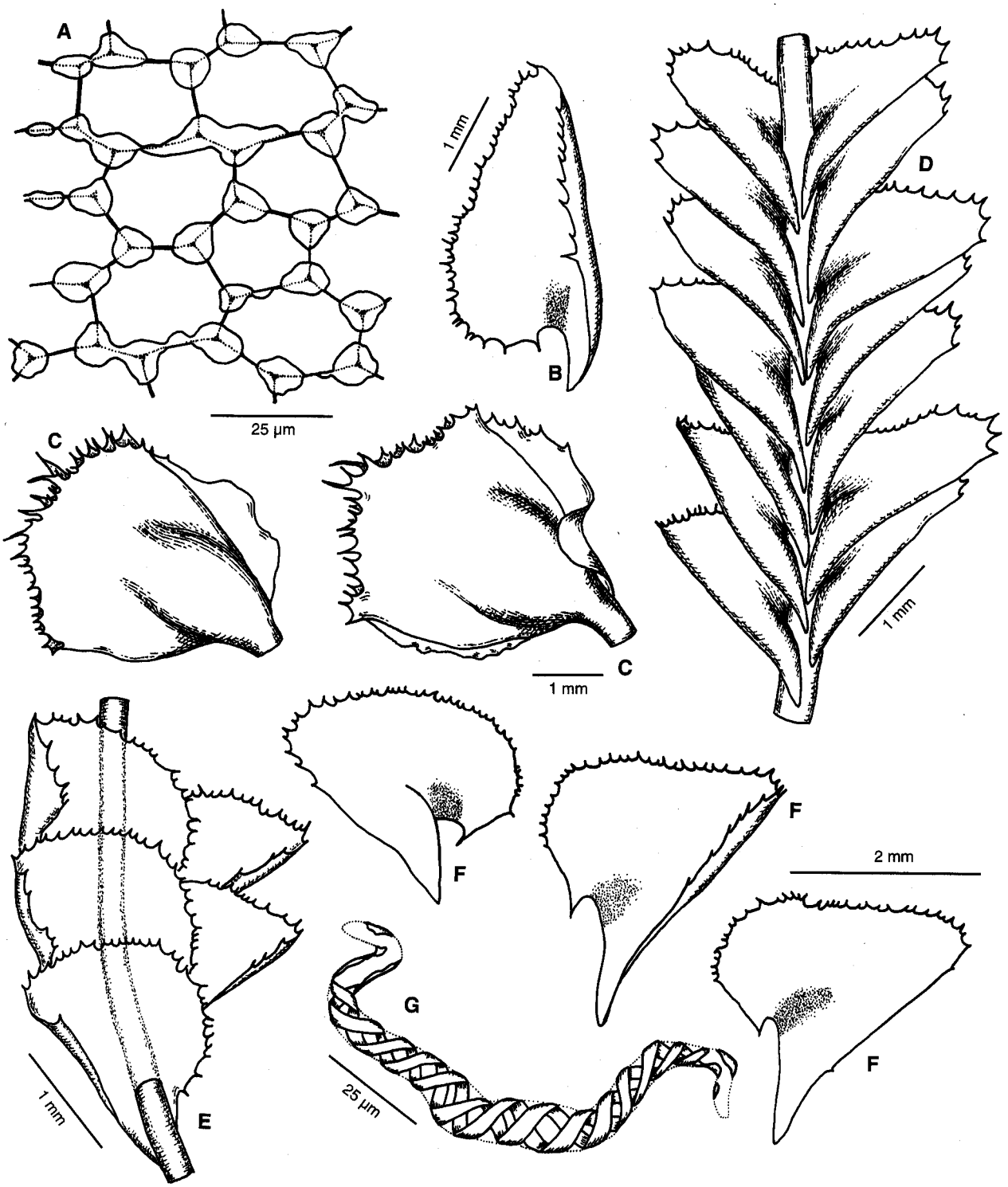

Figure 1. *Plagiochila retrorsa* Gottsche: A, cells from upper leaf half; B, female bract; C, perianths, lateral view; D, part of shoot, dorsal view; E, part of shoot, lateral ventral view; F, leaves; G, elater [all from Costa Rica, *Kappelle & Guttiérez 1844* (GOET)].

smooth or surface slightly irregular, spherical to ellipsoidal, colourless, *ca* (4-)5–8(-12) per median leaf cell, *ca* 4–9 × 4–6 µm (description from Costa Rican and Madeiran plants). *Underleaves* lacking or small, built by few stalked slime papillae, occasionally short cilia present terminated by slime papillae. Asexual reproduction not observed.

Male plants as large as female plants. Androecia becoming intercalary, bracts in *ca* (6-)8–20(-24) pairs, closely imbricate, in the transition zone to leaves sometimes loosely imbricate or remote, opposite bracts overlapping on the dorsal side of the stem; basal part of bracts strongly inflated, mainly composed of hyaline and partly weakly inflated cells with thin walls without

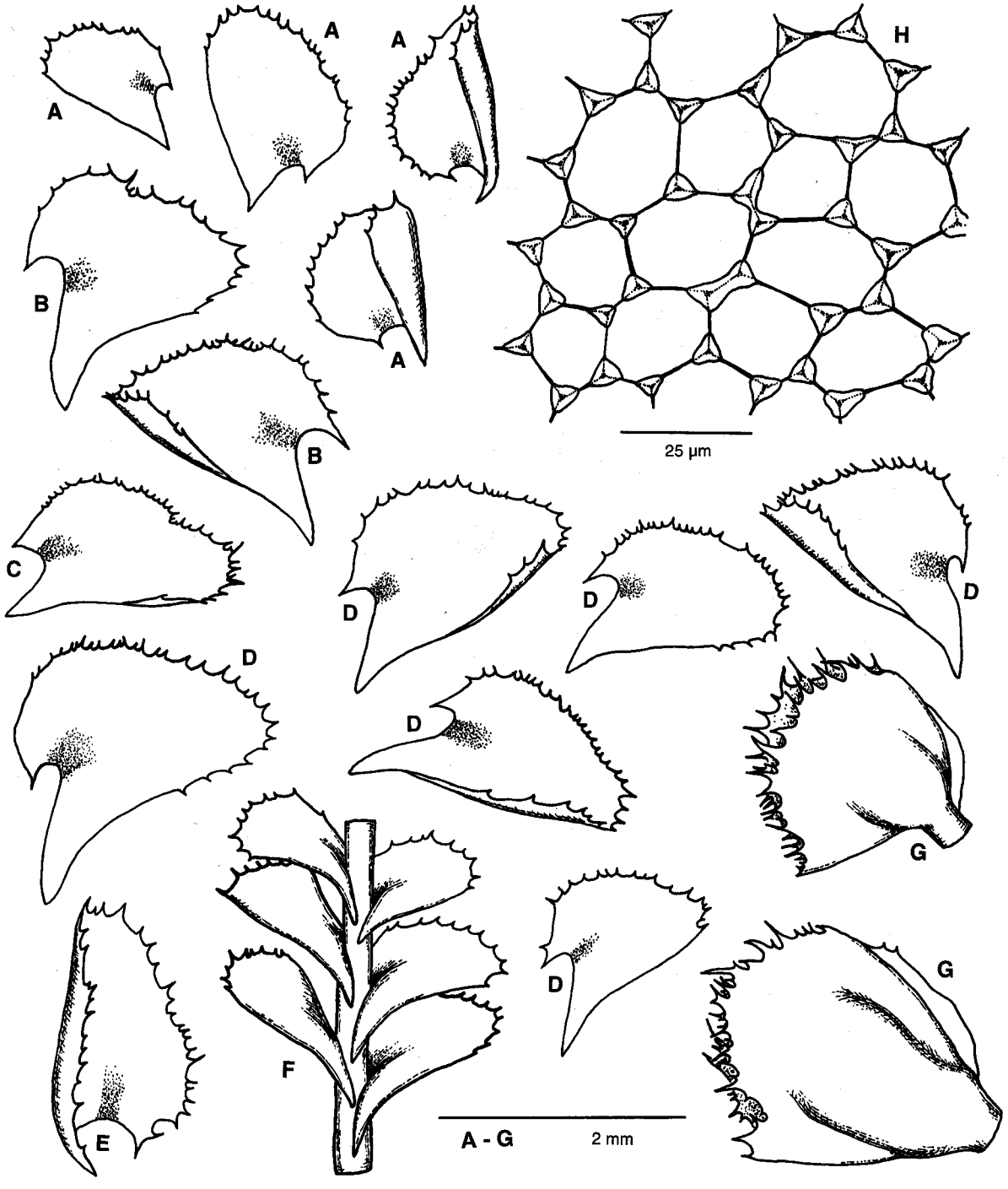

Figure 2. *Plagiochila retrorsa* Gottsche: A–D, leaves; E, female bract; F, part of shoot, dorsal view; G, perianths, lateral view; H, cells from upper leaf half [A from Tennessee, *Sharp 34479* (U), B from Tennessee, *Sharp 34596* (U), C from Tennessee, *Sharp 5647* (U), D–H from Madeira, *Drehwald 2993* (GOET)].

trigones or with small triangular ones; distal part of bracts obliquely spreading, composed of leaf-like cells, margin of bracts with fewer teeth than leaves (usually 2–9), occasionally entire with acute apex and ventral part ± crenulate, dorsal margin of inflated part sometimes with 1–2(-3) ciliate teeth or short cilia often terminated by slime papillae. *Antheridia* (1-)2–4 per bract, on short stalks. *Gynoecia* terminal on branches and on main shoots, 1–2 subgynoecial innovations nearly always present, usually again with gynoecia, innovations originating in axils of bracts or below bracts; bracts often somewhat larger than the subtending leaves, ± similar in shape but with more (to *ca* 52) and stronger elongate teeth (to *ca* 9 cells long), teeth present on complete margin or lacking in lower $\frac{1}{3}$–$\frac{1}{2}$ of dorsal margin, highest density of teeth on ventral margin. *Perianths* in lateral view triangular to campanulate with mouth arched upwards, inflated basally, distinctly narrowed

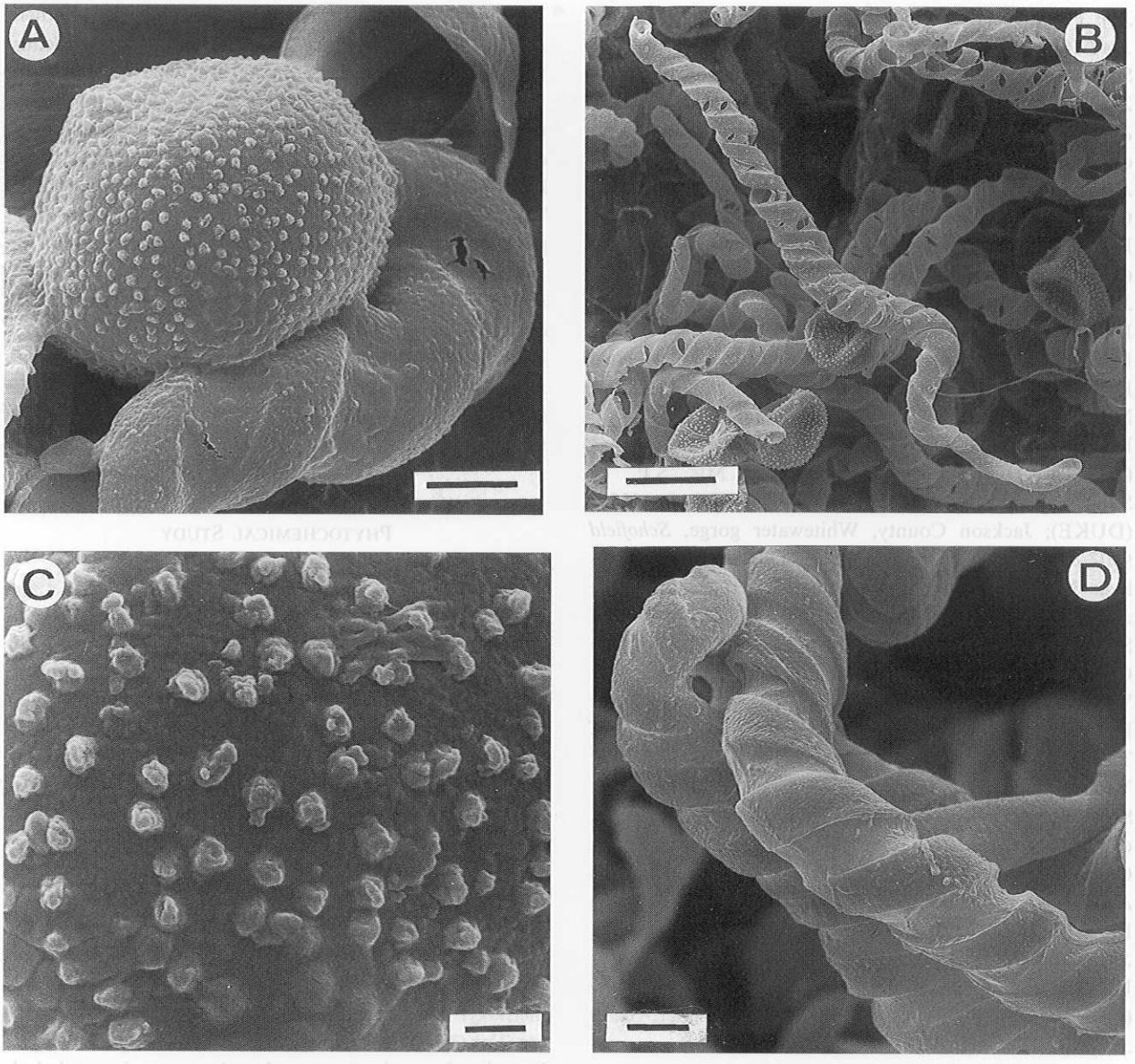

Figure 3. Scanning electron micrographs of *Plagiochila retrorsa* Gottsche: A, spore; B, elater; C, detail of sporoderm; D, parts of elaters [all from Costa Rica, *Kappelle & Guttiérez 1844* (GOET); scale bars: A, D = 5 μm; B = 20 μm; C = 1 μm].

towards mouth in dorsal view, when fully developed *ca* 1.5–3.6 mm long and 1.3–3.5 mm broad, *ca* 0.9–1.1 times as long as wide; dorsal keel slightly longer than ventral one; perianth unwinged or with a low to high, 0–8-toothed, often somewhat undulate dorsal wing, occasionally a second, low, 0–1-toothed ventral wing present (rarely accompanied by accessorial wings); perianth mouth densely beset with large elongate triangular teeth up to *ca* 20 cells long and 16 cells broad, sometimes with 1–3 longer, coarser teeth in median part of mouth (lateral view). *Capsule* [description from valve fragments found in a perianth of *Kappelle & Guttiérez 1844*, Fig. 3] exceeding the perianth only slightly. *Spores* globose, *ca* 20–25 μm in diameter, sporoderm with finely granulate basal surface and rather low, ± densely spaced baculate structures. *Elaters* bispiral, *ca* 9–13 μm in diameter, ± smooth.

Representative specimens examined

Mexico. Chiapas: San Cristobal de Las Casas, N.W. side of Cerro Tzontehuitz, 2830 m, *Breedlove 67303* (MO 3683998). **Costa Rica. Cartago:** Wood behind Hotel La Georgina, 2950 m, *Gradstein Mues 9717* (SAAR); Panamerican Highway 1 km W. of Hotel [La] Georgina and 3 km W. of villa Mills, 3100 m, *Nee & Mori 3532* (MO 2140379); Panamerican Highway (km 78) between Cartago and Cerro de la Muerte, 2900 m, *Heinrichs et al. 4152* (GOET, INB); Cerro de la Muerte, 2600 m, *Gradstein & Mues 9703* (SAAR); Río Savegre valley, Jaboncillo, 2900 m, *Heinrichs et al. 4146* (GOET, INB); Cerros Cuericí, Parque Nacional Chirripó, 3200 m, *Davidse 24827 & 24834* (GOET, U); **San José:** Cerro de la Muerte, Paramo Buena Vista at km 85 of Panamerican Highway, 3100 m, *Heinrichs et al. 4150 & 4151* (G, GOET, INB,

JE); Providencia de Dota, 2880 m, *Kappelle & Guttiérez 1844* (GOET, U); San Gerardo de Dota, road between San Gerardo de Dota and Panamerican Highway, 2900–2920 m, *Kappelle 1675* (GOET, U) & *Heinrichs et al. 4153 & 4154* (GOET, INB); Cerro de Las Vueltas, *ca* 500 m E. of Sitios Garrafa, *Kappelle 2200* (GOET, U). **Portugal. Azores:** Terceira, Terra Brava, *ca* 1 km N.E. of Algar do Carvão, 640 m, *Bates & Gabriel 3478* (hb. Bates); **Madeira:** Levada do Furado between Portela and Ribeiro Frio, 650–750 m, *Drehwald & Reiner-Drehwald 2993* (G, GOET, JE); ibid., 850 m, *Rycroft 99042* (GOET); Rabaçal, Levada do Risco, 1050 m, *Rycroft 99051* (AZU, GOET, MADJ). **U.S.A. Georgia:** Rabun County, 0.3 miles below High Falls of Big Creek, *Schuster 40723* (DUKE, GL); **North Carolina:** Graham County, Nantahala National Forest, Santeetlah Creek, 2800 ft, *Davison & Hicks 2943* (DUKE); Haywood County, Balsam Mts S.E. of Sunburst, 6050 ft, *Anderson 11177, 11178 & 11180* (DUKE); Jackson County, Whitewater gorge, *Schofield 9407 & Schuster 25056* (DUKE); Macon County, Nantahala National Forest, Chattogoga River, 2280–2400 ft, *Hicks & Davison 2284* (DUKE); ibid., Big Creek 8 miles S.E. of Highlands, *Anderson 10454* (DUKE); Transylvania County, Corbin Creek above footlog and confluence with Whitewater, *Hicks s.n.* (DUKE); ibid., Devil's Court House, 6500 ft, *Anderson 11144* (DUKE); ibid., gorge of Thompson River, 1500 ft, *Schuster 45153a* (DUKE); Yancey County, Middle Creek, *Schofield 5949* (DUKE); **South Carolina:** Oconee County, Whitewater River N.W. of Jocassee, *Schuster 25169a* (DUKE); **Tennessee:** Sevier County, Smoky Mt. National Park, 'Chimneys Parking Area', 4000 ft, *Schuster 36541* (DUKE); ibid., Roaring Fork, *Sharp 5647* (GOET, U); ibid., Mt LeConte, 4800 ft, *Sharp s.n.* (DUKE); ibid., 4000 ft, *Sharp 34518* (GOET, U); ibid., near Rainbow Falls, 4500 ft, *Sharp 34479* (GOET, U); ibid., Pinnacle Trail near Greenbrier, *Sharp 34596* (GOET, U).

Distribution and ecology

Plagiochila retrorsa occurs in Central America and the Southern Appalachian mountains of the eastern U.S.A. Additionally, the species is now known from the Azores and Madeira, new to Europe with Macaronesia.

In Central America, *P. retrorsa* occurs at altitudes between 2600 and 3200 m. Specimens were seen from Mexico and Costa Rica. It is very likely that *P. retrorsa* is much more widespread in the high mountains of Central America but the limited number of specimens available does not allow a definitive statement.

P. retrorsa is locally abundant in the Cordillera Talamanca of Costa Rica where it occurs in oak forests (*Quercus copeyensis* C.H.Müll. and *Q. costaricensis* Liebm.) and in shrubby communities of the subparamo. The species grows on the lower parts of tree trunks, on roots and on soil or rock.

In North America, *P. retrorsa* is found in rich Southern Appalachian forests where it often grows on rocks (Schuster, 1980) at altitudes between 500 and 2000 m.

In the Azores and on Madeira *P. retrorsa* grows epiphytically, as well as on rocks, in humid parts of laurel forests, dominated by *Laurus azorica* (Seub.) Franco. All the known stations are in steep north-facing forests or woods. The habitat on Terceira (Azores) with respect to the bryophyte flora has been described by Bates & Gabriel (1997).

The occurrence of *P. retrorsa* [sub *P. sharpii* ssp. *yakusimensis* (S.Hatt.) R.M.Schust. = *P. magna* Inoue] in southeastern Asia (Schuster, 1959, 1980) remains doubtful. As a rule, rhizoids are totally absent on aerial shoots of *P. retrorsa* s.str. In contrast, the aerial shoots of Asian plants are covered with numerous rhizoids (Inoue, 1965) indicating that a different species is at hand.

Phytochemical Study

NMR and GC–MS fingerprinting

The 15 most intense components observed in the GC–MS of the 12 plant extracts studied are presented in Table 1. As well as the seven major components, many minor components were detected in the extracts, but the number has been restricted to eight in Table 1 using the criterion given in footnote k. The ^{1}H NMR spectra of the same extracts are shown in Fig. 5. Unlike the situation with *P. bifaria* (Rycroft *et al.*, 1999a) where the ^{1}H NMR spectra are dominated by signals from methyl everninate (**1**) (emboldened numerals refer to the chemical structures shown in Fig. 4), in this case (with one exception) the methoxyl region of the NMR fingerprints (Fig. 5) is dominated by two singlets of equal intensity from the two methoxyl groups in 3,5-dimethoxy-9,10-dihydrophenanthren-2-ol (**2**) (Anton *et al.*, 1997) and two singlets of equal intensity from the two methoxyl groups in methyl 4-hydroxy-4′-*O*-methyllunularate (**3**) (Connolly *et al.*, 1999). The GC–MS TIC (total ion current) integration ratios agree well with the relative amounts of compounds **2** and **3** derived from the NMR spectra.

The presence of compounds **13–16** was confirmed by comparison using GC–MS with reference samples of the compounds available from the earlier work. Compound assignments in Table 1 were supported by observation of peaks in the NMR spectra (except for fusicoccadiene, **5**, considered later).

Compound **12** was not isolated, but is isomeric with **14** and has a very similar mass spectrum. It has been assigned the structure 4-methoxy-9,10-dihydrophenanthrene-3,5-diol on the basis that the ^{1}H NMR spectra of the extracts display a methoxyl singlet at δ 3.69 p.p.m. and a phenolic hydroxyl singlet (exchangeable with D_2O) at δ 8.24 p.p.m. with intensities that correlate with the relative abundances indicated by GC–MS. The methoxyl chemical shift is consistent with a methoxyl substituent at position 4 that

Table 1. *GC–MS TIC (total ion current) integration, of components[a] in $CDCl_3$ extracts of twelve collections of* Plagiochila retrorsa *Gottsche.*

						Extract no. (i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)	(ix)	(x)	(xi)	(xii)
						Country, collection no. and year of collection											
			GC–MS			Madeira			USA			Costa Rica					Mexico
Peak no.[b]	Assignment[c]	Compound no.[d]	R_i[e]	M_r[f]	Base peak	Drehwald 2993 1996	Rycroft 99042 1999	Rycroft 99051 1999	Anderson 10454 1951	Schuster 40723 1953	Hicks *s.n.* 16/7/94 1994	K & G 1844[g] 1986	G & M 9703[h] 1994	G & M 9717[i] 1994	JH 4146 1999	JH 4154 1999	Breedlove 67303 1988
	(a) Major components[j]																
3	β-Phellandrene	**6**	1017	136	93	41	54	43	5	6	38	+	24	25	18	40	16
4	Peculiaroxide	**7**	1390	222	137	4	4	5	15	17	+	+	6	10	6	10	15
6	Bicyclogermacrene	**8**	1476	204	121	18	20	16	–	–	20	–	+	+	16	18	–
7	Spathulenol	**9**	1543	220	205	–	+	2	19	6	13	1	17	19	1	2	7
8	Fusicoccadiene	**5**	1958	272	135	6	+	3	5	8	8	17	5	5	1	16	6
10	3,5-$(MeO)_2$-9,10-dihydrophenanthren-2-ol	**2**	2212	256	256	100	100	100	100	100	100	14	100	100	100	100	100
10-Ac			*2361*	*298*	*256*	*100*	*100*	*100*	*100*	*100*	*100*	*14*	*100*	*100*	*100*	*100*	*100*
14	Methyl 4-hydroxy-4′-*O*-methyllunularate	**3**	2338	302	121	–	61	57	41	–	95	–	18	27	17	9	18
14-Ac_2			*2610*	*386*	*121*	*121*	*158*	*145*	*65*	*71*	*168*	*2*	*31*	*42*	*38*	*32*	*41*
	(b) Minor components[k]																
1	α-Terpinene	**10**	1007	136	121	–	+	+	–	–	–	–	–	–	4	9	–
2	*p*-Cymene	**11**	1011	134	119	–	+	+	+	+	+	–	5	4	+	+	3
5	-		1418	204	133	–	4	6	–	–	–	–	–	–	–	–	–
9	4-MeO-9,10-dihydrophenananthrene-3,5-diol	**12**	2022	242	242	–	+	2	4	–	+	–	–	–	–	–	6
9-Ac_2			*2207*	*326*	*242*	*2*	*4*	*3*	*3*	*3*	*2*	–	–	–	–	–	*11*
11	2,3,5-$(MeO)_3$-9,10-dihydrophenanthrene	**13**	2254	270	270	3	+	5	7	+	+	1	+	+	1	+	+
12	3-MeO-9,10-dihydrophenanthrene-4,5-diol	**14**	2257	242	242	–	–	–	–	–	–	–	4	2	4	5	–
12-Ac_2			*2222*	*326*	*242*	–	–	–	–	–	–	–	*2*	*2*	*7*	*10*	–
13	3,7-$(MeO)_2$-9,10-dihydrophenanthren-2-ol	**15**	2320	256	256	–	+	5	6	+	+	–	3	3	3	3	+
13-Ac			*2473*	*298*	*256*	*3*	*4*	*6*	*4*	*3*	*5*	–	*3*	*3*	*3*	*3*	*2*
15	2,3,5,7-$(MeO)_4$-9,10-dihydrophenanthrene	**16**	2505	300	300	3	+	4	13	–	9	1	2	+	+	+	+

[a] Integration is relative to 100 for peak 10, except for the extract from the Costa Rican sample K & G 1844 (see footnote g). Peaks detectable at a low level are indicated by +. Fatty acids are not included.

[b] Peaks are numbered in order of increasing elution time. The suffix *Ac* indicates peaks in the acetylated extracts where the retention time changed.

[c] Some names are abbreviated: MeO = methoxy.

[d] Compound numbers refer to the structures in Fig. 4.

[e] Kovát's retention index from GC.

[f] Relative molecular mass (*m/z* of parent ion).

[g] In the ^1H NMR spectrum, the molar ratio of compound **2** to **4** (the didehydrodimer of **2**, that is not observed by GC–MS) is 1:3. The abundances for the compounds of this extract have therefore been given relative to 14.3 for peak 10, equivalent to 100 {$= 14.3 \times [1 + (3 \times 2)]$} for total compound-**2**-derived material.

[h] From a duplicate of sample A in Anton *et al.* (1997).

[i] From a duplicate of sample B in Anton *et al.* (1997).

[j] Components > 15% of peak 10 in at least one extract.

[k] Components < 15% in all extracts but ⩾ 4% of peak 10 in at least two extracts.

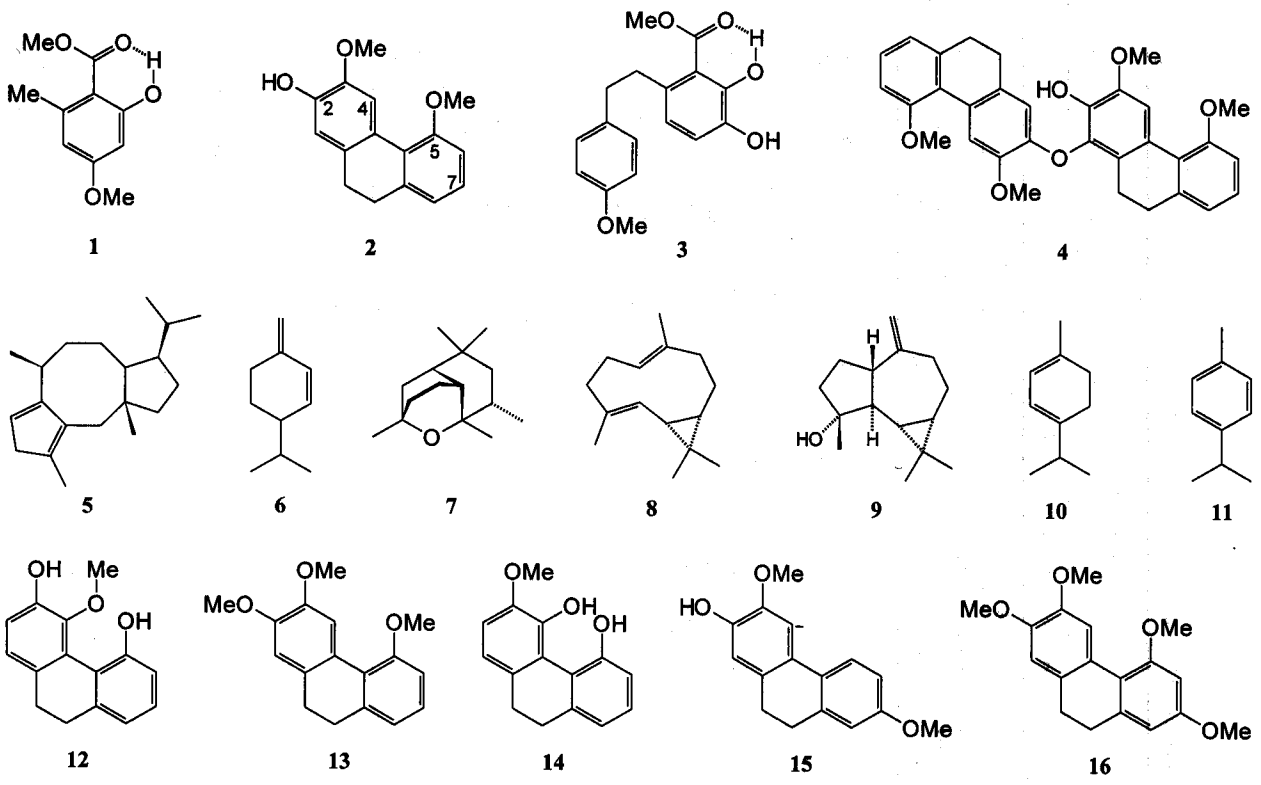

Figure 4. Chemical structures, numbered to coincide with the emboldened compound numbers used in the text.

has a substituent (in this case a hydroxyl group) *ortho* at position 3 (Connolly *et al.*, 1999). The hydroxyl signal is assigned to a second hydroxyl substituent, placed at position 5 because there is no other deshielded signal, as would arise from a proton at position 5. The two NMR signals observed for compound **12** are very similar to those reported for 5,6-dimethoxy-9,10-dihydrophenanthren-4-ol from *Riccardia jackii* Schiffn. (Matsuo *et al.*, 1985). The diacetates of **12** and **14** have similar retention times, whereas the diol **14** has the unusual property of a longer retention time than that of its diacetate. This is attributed to the chelating hydrogen bonding ability of the diol function of **14**, a feature not present in **12**.

The extract with the NMR fingerprint that is conspicuously different from the others is number (vii), K&G 1844. The methoxyl signals of **2** are still present, but the major signals are a set of four methoxyl singlets from a compound that was isolated chromatographically using a small plug of silica gel and shown to be 1-(3,5-dimethoxy-9,10-dihydrophenanthren-2-oxy)-3,5-dimethoxy-9,10-dihydrophenanthren-2-ol (**4**), a didehydrodimer of **2**. Details of the structural elucidation and synthesis of **4** from **2** will be reported elsewhere. The oxidative phenolic coupling that has apparently produced **4** from **2** in the liverwort may well have occurred *post mortem* rather than *in vivo*. The difference shown by this extract loses significance as soon as it is realised that the major component can still be regarded as derived from **2**. Similarly, minor methoxyl signals at 3.868 and 3.785 p.p.m. along with, *inter alia*, a doublet at δ 6.56 p.p.m. can be assigned to spinuloplagin A (Rycroft, 1990; Srivastava, 1995), a product of oxidative addition of **3** to fusicoccadiene (**5**).

Secondary metabolite characters

Except for compounds **2** and **3**, the major components observed in the extracts (Table 1) occur commonly in liverworts (Asakawa, 1995). β-Phellandrene (**6**) is a relatively volatile monoterpene, yet considerable amounts are still present in samples nearly 50 years old. Peculiaroxide (**7**; Wu, Huang & Shih, 1993) is characterized by a relatively short GC retention time, attributable to its unusually compact structure, and is present in several *Plagiochila* species, including *P. punctata* Taylor, *P. spinulosa* (Dicks.) Dumort. and *P. bifaria*, where one of the previously unidentified peaks (no. 2 of Table 1 in Rycroft *et al.*, 1999a) can now be assigned to this compound. In addition, GC–MS results reported for extracts of four *Plagiochila* species from Chile (Asakawa & Inoue, 1984) and nine from Peru (Asakawa & Inoue, 1987) contain an unassigned peak with M_r 222 and base peak 137, that elutes relatively early and is likely to arise from peculiaroxide. It has also been found in *P. dusenii* Steph. and *P. validissima* Steph. (Anton *et al.*, 2000). Bicyclogermacrene (**8**) should be considered in conjunction with spathulenol (**9**) as Toyota *et al.* (1996) have shown that spathulenol can occur as an artefact arising from oxidation of bicyclogermacrene; our data suggest that this is also the case here, as the most recent samples contain large

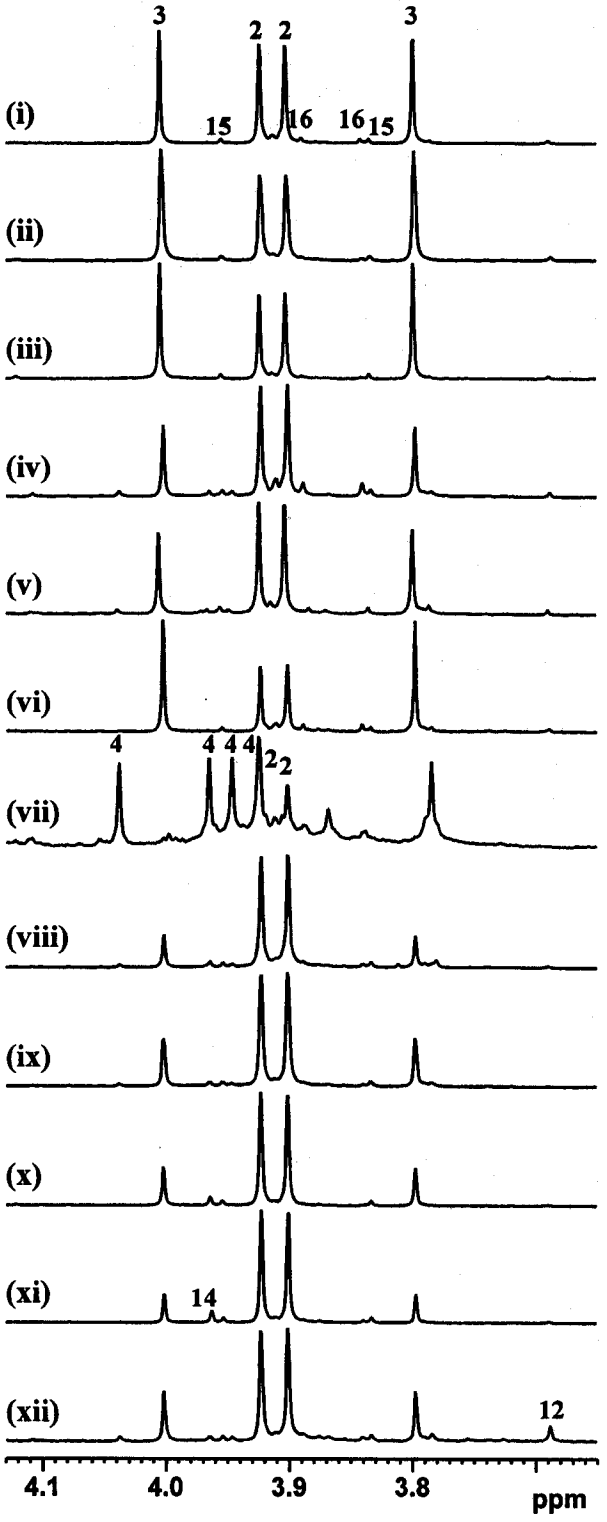

Figure 5. The methoxyl region of the 400 MHz ^{1}H NMR spectra of twelve $CDCl_3$ extracts of *Plagiochila retrorsa* Gottsche. The numbers (i)–(xii) refer to the extracts in Table 1. The peak labels refer to compound numbers in Fig. 4 and Table 1; for clarity and/or because of overlap, not all peaks of all compounds are labelled.

amounts of bicyclogermacrene and little spathulenol, while the oldest samples contain spathulenol but no bicyclogermacrene. Fusicoccadiene (**5**) is a diterpene that has been reported in several liverwort extracts (e.g. Zapp, Burkhardt & Becker, 1994), but its presence in a GC–MS chromatogram can also arise because a retro-Diels–Alder reaction has occurred at the high temperature of the inlet port of the chromatography column (Spörle *et al.*, 1989); several different adducts might decompose thermally to produce **5** and this may explain why no signals of **5** (Kato *et al.*, 1994) or of a single, abundant precursor were observed in the NMR spectra of the *P. retrorsa* extracts.

Among the minor components there are two monoterpenoids. The occurrence (in other than trace amounts) of α-terpinene (**10**) in the two 1999 Costa Rican samples (extracted within 2 months of collection) and of *p*-cymene (**11**) in the two 1994 Costa Rican samples suggests the possibility that **11** arises from oxidation (dehydrogenation) of **10** *post mortem*. Peak 5 is an unidentified sesquiterpene that is present only in the two 1999 Madeiran samples (extracted within two weeks of collection). The five remaining minor components in Table 1 are 9,10-dihydrophenanthrenes: compounds **13–16** were reported previously by Anton *et al.* (1997) from the Costa Rican *Plagiochila* now identified as *P. retrorsa* whereas compound **12** appears to be new.

Of the remaining compounds reported by Anton *et al.* (1997), 3,5-dimethoxyphenanthren-2-ol (formally derived by dehydrogenation of compound **2**) was detected in seven extracts, with the largest amount (4% of compound **2**) observed in the oldest extract. 4-Hydroxy-3′-methoxybibenzyl, the compound isolated in the third largest amount by Anton *et al.* (1997), was detected (at the level of 4% of compound **2**) in only one (G&M 9703) of the two vouchers from the previous work and in none of the other samples. A possible explanation of this most surprising result would be that the larger amount of material processed in the previous work included a small amount of a liverwort containing a high level of 4-hydroxy-3′-methoxybibenzyl. Several specimens of such a plant, an unidentified species of *Plagiochila cf.* sect. *Contiguae* Carl (det. JH), were found by DSR in 1999 on Madeira growing in similar sites to *P. retrorsa* and *P. bifaria*; the level of 4-hydroxy-3′-methoxybibenzyl was *ca* 6% w/w (dry weight). As the level of compound **2** found in G&M 9703 was *ca* 0.8% w/w, the presence of only 0.5% of such a liverwort in the sample of G&M 9703 extracted for the NMR fingerprint would account for the amount of 4-hydroxy-3′-methoxybibenzyl observed in extract (viii); *ca* 4% (i.e. only one shoot in 25) would have had to have been present in the bulk sample extracted previously to account for the amount of 4-hydroxy-3′-methoxybibenzyl isolated.

The bisbibenzyls isolated previously would not be detected under the GC–MS conditions used here and, at the low levels involved, they would not form distinctive features in the NMR spectra.

Discussion

Morphology and synonymy

Morphological variation of *Plagiochila retrorsa* is considerable with respect to plant size and leaf dentition. North American and European plants (Fig. 2) are small to medium sized with shoots as a rule not longer than 40(50) mm. Central American plants (Fig. 1) are usually larger, producing shoots up to more than 100 mm in length. Luxuriant Central American phenotypes are often provided with larger leaves and perianths than those found in plants from temperate regions. Despite Gottsche (1863) using the description 'sesquipollicaris' (4 cm long), Blomquist (1940) considered *P. retrorsa* to possess stems 'ranging from 10 to 15 cm' and proposed *P. sharpii* as a new species because of the generally smaller size ('stems 2–3 cm long'). However, the extremes are combined by intermediate forms and variation between Holarctic stands is as high as between Neotropical populations. Furthermore, size should not be the only feature used to distinguish two hepatic species. Such variation is not unusual and is well known in Britain in *P. spinulosa*, one of the species considered by Blomquist to resemble *P. sharpii*.

Neotropical plants are sometimes provided with perianths possessing a dorsal and a ventral wing (Fig. 1, C). *P. tricarinata* from Costa Rica was described as a new species mainly because of the presence of a second, ventral wing (Carl, 1931; Herzog, 1932). Plants from the Holarctic usually have only a dorsal wing. However, a low ventral wing was recognized once in a North American specimen (*Anderson 10454*, DUKE). Sporophytes were recognized only in Neotropical gatherings, indicating that the species grows in the Holarctic under suboptimal conditions.

Differentiation

Plagiochila retrorsa stands out by the postically secund leaves with a smooth cuticle and distinct vitta and the short, triangular to campanulate perianths. In the U.S.A. there has sometimes been confusion with *P. porelloides* (Torr. ex Nees) Lindenb. (Schuster, 1980), but within European *Plagiochilae* the species is most likely to be confused with *P. spinulosa* and *P. bifaria*. However, a vitta is lacking in the leaves of *P. spinulosa*. Leaves of *P. bifaria* usually possess a ± entire dorsal leaf margin whereas the leaves of *P. retrorsa* are often toothed dorsally, especially in the upper half. The presence normally of relatively more teeth on the ventral leaf margin of *P. retrorsa* compared to *P. bifaria* is also distinctive.

Phytochemistry

Regional differences in the composition of the extracts are relatively small. There is less of compound **3** relative to **2** in the Central American populations compared to the others. Amongst the minor components the most obvious differences are the absence of the diol **12** from all the Costa Rican specimens and the presence of the diol **14** in the four most recent Costa Rican specimens only.

None of the secondary metabolites observed is useful as a single character that can define *P. retrorsa* unambiguously. Neither of the two major components is unique to *P. retrorsa*: the 9,10-dihydrophenanthrene **2** is a minor component in *P. bifaria* (Rycroft *et al.*, 1999a) and the lunularic acid derivative **3** is a major component in *P. spinulosa* (Connolly *et al.*, 1999). The combination of **2** and **3** observed (Fig. 5) is variable, but appears at first sight to be able to distinguish *P. retrorsa* from other *Plagiochila* species that have been studied, for example, the British *Plagiochilae* (Rycroft, 1999). However, during the course of this work several other Neotropical and Macaronesian specimens were investigated. Two of them (both so far unidentified: *Plagiochila* sect. *Arrectae*, coll. Anton, Heinrichs & Müller, BOL GP4, Bolivia, 7 October 1997; *Plagiochila* sp., coll. Dias *s.n.*, Pico, Azores, 9 July 1992) had NMR fingerprints very similar to those of *P. retrorsa*. In addition, the NMR fingerprints, taken as a whole, of these two extracts fit within the range of variation displayed by extracts (i)–(xii), even when the minor components are considered. It is therefore clear that NMR fingerprints typical of *P. retrorsa* can also be displayed by other species and that identification of *P. retrorsa* must rely on study of the morphology.

Phytogeography

Phytogeographical relationships between the American and European bryophyte floras have been of interest for a long time. Indeed, Sharp (1941) was writing about the significant geographical affinities at around the same time as Blomquist (1940) was publishing *P. sharpii*. Cronk (1992) has argued in the case of the vascular plant flora that all disseminules must have arrived in Macaronesia from Africa, whereas Schuster (1983) has considered a wider range of possibilities for bryophytes. Sérgio (1984) treated the geographical elements of the Macaronesian bryophytes largely along the lines of the vascular plant flora (Sunding, 1979); she also considered possible migration routes during the Tertiary, but emphasized that generalization is difficult as each distribution event has to be considered independently. As with the systematic questions considered below, unresolved problems concerning populations separated by continental drift and long range dispersal and establishment of species on isolated and relatively recent volcanic islands, as exemplified by the Azores and Madeira, may eventually be solved by analysis of DNA sequences.

The known range of *P. retrorsa* is somewhat similar to that attributed to *P. dubia* Lindenb. & Gottsche, a

Neotropical *Plagiochila* that was added to the Macaronesian bryophyte flora some years ago (Dirkse, Bouman & Losada-Lima, 1993; Nieuwkoop and Arts, 1995). Together with *P. longispina* Lindenb. & Gottsche (Heinrichs *et al.*, 2000), *P. bifaria* and *P. exigua* (Taylor) Taylor there may now be five *Plagiochilae* that straddle the Atlantic Ocean from the Neotropics to Europe, so that this situation, formerly considered unusual in the genus *Plagiochila*, might now be regarded as unexceptional.

Systematics

The systematic position of *Plagiochila retrorsa* remains unclear. Carl (1931) included the species in his sect. *Permistae*, containing a group of Neotropical species with postically secund leaves. Inoue (1965) included *P. retrorsa* (sub *P. sharpii*) in his '*Plagiochila semidecurrens*-complex' indicating that the closest relatives of *P. retrorsa* occur in Asia. This opinion was supported by Schuster (1959; 1980) who placed the species (sub *P. sharpii*) in the sect. *Zonatae* with species of mainly Asiatic distribution. The low baculate structures on the spores (Fig. 3, A & C) and the more or less smooth surface of the bispiral elaters (Fig. 3, B & D) differ from the high baculate structures on the spores and the rough surface of the mostly unispiral elaters observed in members of sect. *Arrectae* Carl such as *P. bifaria*. Unfortunately, the equivalent ultrastructural knowledge for sect. *Zonatae* is lacking.

Chemosystematically, *P. retrorsa* belongs to the 9,10-dihydrophenanthrene chemotype group (Rycroft, 1998b; Rycroft *et al.*, 1999a), members of which are so far known only from the Neotropics and Europe (Rycroft, 1999). This is in contrast to *P. semidecurrens* and *P. magna*, that both contain 2,3-secoaromadendranes (Asakawa *et al.*, 1980).

The range extensions reported here, although significant in terms of the origins of the European bryoflora, do not add directly to knowledge of the systematic position. More information, including molecular (DNA) data, is needed to decide on the sectional membership of *P. retrorsa*. Work to obtain these data is underway.

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TAXONOMIC ADDITIONS AND CHANGES: *Plagiochila retrorsa* Gottsche (syn. *P. tricarinata* Carl, *P. permista* Spruce var. *subintegerrima* Herzog, *P. sharpii* H.L.Blomq.).

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