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Comparative molecular and phytochemical investigation of *Leontodon autumnalis* (Asteraceae, Lactuceae) populations from Central Europe

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Abstract

Previous analyses of *Leontodon autumnalis* L. revealed the existence of two chemotypes. In the current study molecular and phytochemical methods were combined to investigate 24 Central European populations of *L. autumnalis.* The focus of this study was the correlation of molecular and phytochemical characters at the intraspecific level. DNA fingerprint profiles of 183 individuals were obtained by random amplified polymorphic DNA (RAPD) providing 77 molecular markers. Contents of phenolics and sesquiterpenoids of flowering heads and subaerial parts were quantified by HPLC-DAD analyses. HPLC results were evaluated by principal component analysis. Geographic distribution of the two detected chemotypes partially overlapped. Phylogenetic groupings displayed in an unrooted neighbor-joining tree calculated from the RAPD data matrix were correlated with the geographical origin of the plant material. However, genetic profiles neither correlated with the two chemotypes nor with the morphologically based subspecies of *L. autumnalis* recognized by some authors. The presented data imply that the morphotypes are of multiple origins or due to different ecological growing conditions rather than genetically determined and that phytochemical races are induced by a limited number of genetical differences, which might have occurred independently in different lineages of the *L. autumnalis* group.

Keywords: *Leontodon autumnalis*; Asteraceae; Lactuceae; Chemosystematics; Molecular biology; Random amplified polymorphic DNA; Sesquiterpenoids; Phenolics

1. Introduction

Both, molecular phylogenetic studies and studies of the secondary metabolite profile of higher plants have received considerable attention in recent years. However, with some notable exceptions (e.g., Adams, 1999, 2001; Baum et al., 2001; Fico et al., 2003; Johnson et al., 2003; Vieira et al., 2001) studies combining molecular biology and phytochemistry, which might give valuable insights into the role of secondary metabolites and the onset of phytochemical differentiation in evolution, are almost lacking.

In our continuing investigation of the phytochemistry and phylogeny of the Lactuceae tribe of the Asteraceae family, we have reinvestigated *Leontodon autumnalis* L., a perennial herb, which is distributed over most of Europe – except for the far South and South-East – to Western Siberia (Meusel and Jäger, 1992). Based on morphological and ecological characters, *L. autumnalis* is subdivided in a number of subspecies by many authors (Finch and Sell, 1976). However, currently no satisfying intraspecific system for the whole distribution range of the species is available and many of the described subspecies and variations seem to be merely based on modifications without systematic significance. In addition to the morphologic variation, a previous study revealed the existence of two different chemotypes of *L. autumnalis*. One chemotype contained a

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more complex spectrum of sesquiterpenoids, crepidiasides A and B as well as a number of their dihydroderivatives, than the second, which only contained crepidiasides A and B in higher quantities. The former was prevalent in North-Western Central Europe, while the latter chemotype was restricted to the alpine populations investigated (Zidorn et al., 2000). Additionally, the two chemotypes differed in their quantitative spectrum of phenolic compounds and in their response to elevated UV-B radiation in higher altitude sites. The North-Western type showed a more pronounced increase of luteolin-type flavonoids with altitude than the chemotype, which was prevalent in the Alps (Zidorn and Stuppner, 2001a). In the present communication we investigate the correlation of anonymous molecular markers and the two observed chemotypes.

2. Results

The structures of flavonoids, phenolic acids, and sesquiterpenoids detected in extracts of *L. autumnalis* are depicted in Fig. 1. Figs. 2a and 2b show example chromatograms obtained in the analysis of sesquiterpene lactones in subaerial parts of *L. autumnalis* (Fig. 2a) and phenolic compounds in flowering heads (Fig. 2b), respectively. Table 1 gives an overview of the investigated samples and their geographic origin. HPLC quantification results for phenolic compounds and sesquiterpenoids are summarized in Tables 2 and 3, respectively. Absolute HPLC quantification results for all classes of compounds investigated (flavonoids, phenolic acids, and sesquiterpenoids) were analyzed by principal component analysis (PCA). The PCA resulted in two weakly separated clusters, representing chemotypes A and B (Fig. 3). In accordance with previous studies (Zidorn and Stuppner, 2001a,b; Zidorn et al., 2002, 2005), a PCA performed with relative quantification results (i.e., representing each particular compound as a percentage of the total content of its compound class–flavonoid, phenolic acid or sesquiterpenoid, respectively) resulted in a much sharper separation of the two observed chemotypes (Fig. 4). An investigation of the loadings of the particular compounds (data not shown) indicated that the separation of the two chemotypes is mainly based on the relative amounts of sesquiterpenoids present in extracts of subaerial parts of *L. autumnalis*. This fact is highlighted by the result of another PCA solely based on the relative amounts of sesquiterpenoids **S1**–**S6** (Fig. 5). The underlying differences are also indicated in Fig. 2a, showing HPL chromatograms of extracts of chemotype A and B. The geographic origin of the investigated plant material and the assignment of these populations to chemotypes A and B are visualized in Fig. 6. Populations from the Alps exclusively belong to chemotype A, whereas extra-alpine populations either belong to chemotype A or B. In detail, populations 1, 3–12, 16, and 19–23 belonged to chemotype A and the remaining populations (2, 13, 14, 15, 17, 18, and 24) were assigned to chemotype B.

In a second step of our investigation, genetic variability within and between the 24 populations were assessed by RAPD analysis employing sixteen different primers (Table

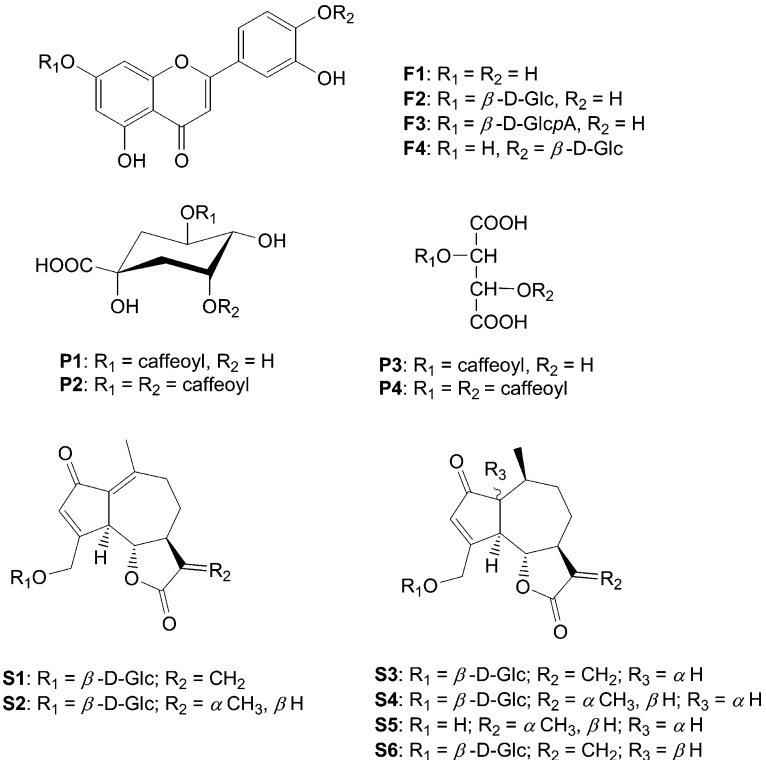

Fig. 1. Structures of investigated flavonoids (**F1**–**F4**), phenolic acids (**P1**–**P4**), and sesquiterpenoids (**S1**–**S6**).

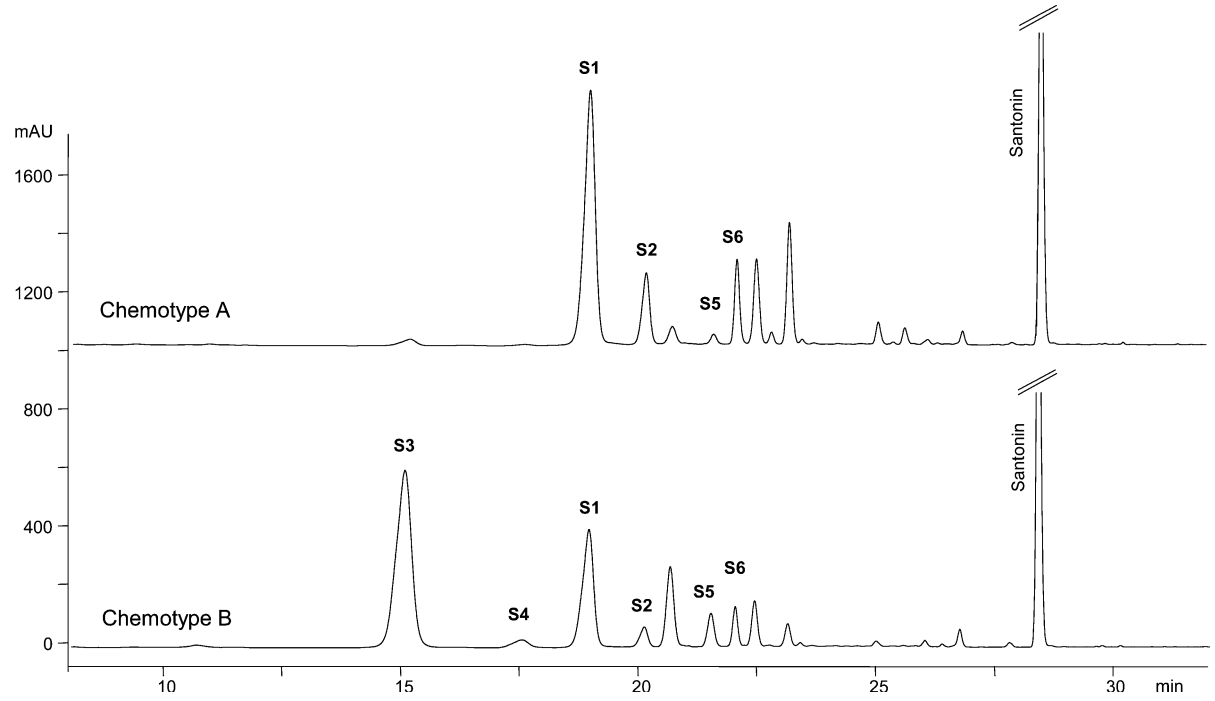

Fig. 2a. HPL chromatograms of methanolic extracts from subaerial parts of *Leontodon autumnalis* chemotypes A and B. HPLC system: column, Zorbax SB-C18, 4.6 × 150 mm, particle size 3.5 μm; guard column, LiChrospher 100 RP-18 4 × 4 mm material (5 μm particle size); mobile phase A, H_2O; mobile phase B, CH_3CN; linear gradient from 12% B to 15% B in 15 min, 25% B in 10 min, 60% B in 5 min, and 98% B in another 5 min, stop time 45 min, post time 12 min; flow rate 1.00 ml/min; detection wavelength, 245 nm; injection volume, 10 μl; oven temperature, 40 °C. **S1** = crepidiaside A, **S2** = crepidiaside B, **S3** = 15-glucopyranosyloxy-2-oxo-guaia-3,11(13)-dien-1α,5α,6β,7α,10αH-12,6-olide, **S4** = 15-glucopyranosyloxy-2-oxo-guaia-3-en-1α,5α,6β,7α,10α,11βH-12,6-olide, **S5** = 15-hydroxy-2-oxo-guaia-3-en-1α,5α,6β,7α,10α,11βH-12,6-olide, **S6** = 15-glucopyranosyloxy-2-oxo-guaia-3,11(13)-dien-1β,5α,6β,7α,10αH-12,6-olide.

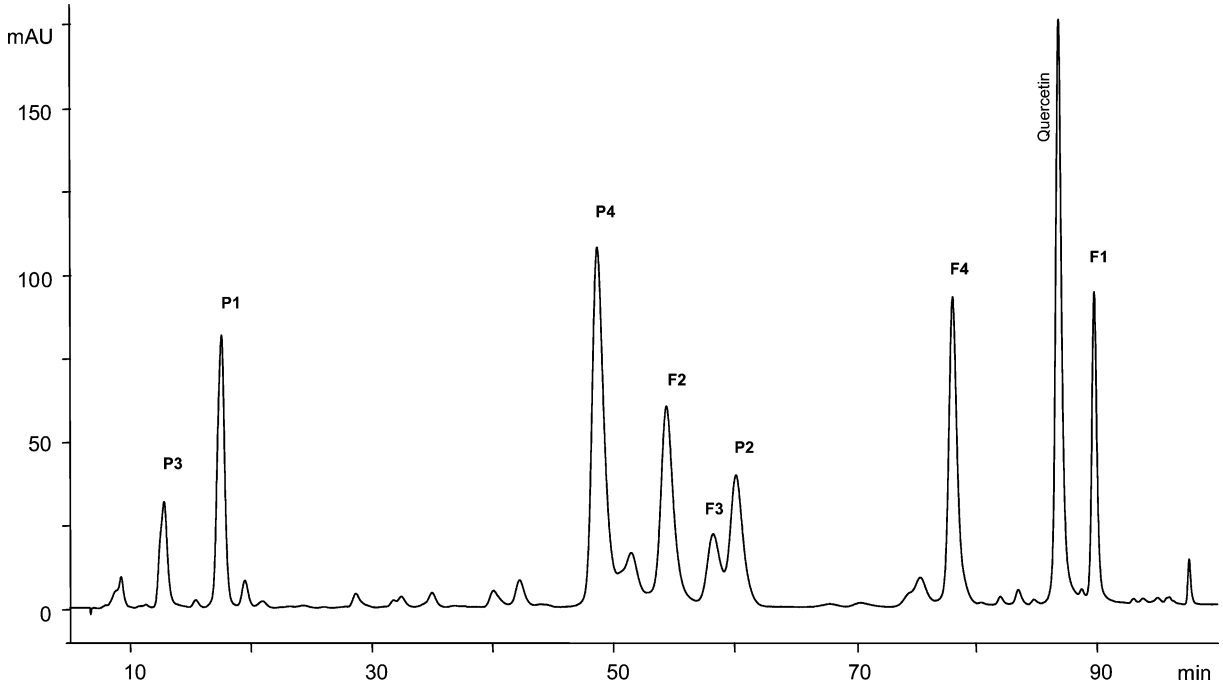

Fig. 2b. HPL chromatogram of a $MeOH/Me_2CO/H_2O$ extract from flowering heads of *Leontodon autumnalis*. HPLC system: column, HP-ODS-Hypersil, 2.1 mm × 200 mm, particle size 5 μm; guard column, LiChrospher 100 RP-18 material 4 × 4 mm, 5 μm particle size; mobile phase A, 0.1% trifluoroacetic acid and 0.5% tetrahydrofuran in water; mobile phase B, 0.1% trifluoroacetic acid and 0.5% tetrahydrofuran in MeOH; linear gradient: 0 min 85% A, 15% B; 25 min 72.5% A, 27.5% B; 55 min 69.5% A, 30.5% B; 65 min 67.5% A, 32.5% B; 95 min 35% A, 65% B; stop time, 100 min; post time, 15 min; flow rate, 0.220 ml/min; injection volume, 5 μl; detection wavelength, 350 nm. **F1** = luteolin, **F2** = luteolin 7-*O*-β-D-glucoside, **F3** = luteolin 7-*O*-β-D-glucuronide, **F4** = luteolin 4′-*O*-β-D-glucoside, **P1** = chlorogenic acid, **P2** = 3,5-dicaffeoyl quinic acid, **P3** = caffeoyl tartaric acid, **P4** = cichoric acid.

Table 1
Origin of plant material

Accession no.	Voucher code[a]	Collection site	Altitude (m)	Coordinates
1	20030615-B1	I/TN/Ultental, between Simian and Riemerbergl Alm	1880	N 46°32′; E 10°57′
2	20030707-E1	GB/Devon/Dartmoor, Princetown	430	N 50°32′; W 03°59′
3	20030708-C1	GB/Greater London/Brentford, London	10	N 51°29′; W 00°17′
4	20030718-A1	I/AO, Val de Veny	1970	N 45°46′; E 06°51′
5	20030719-A1	I/AO, between Rifugio A. Deffeyes and Passo Alto	2480	N 45°40′; E 06°59′
6	20030719-F1	I/AO, Rifugio A. Deffeyes	2480	N 45°40′; E 06°59′
7	20030728-A1	I/TN/Ritten, between Maria Himmelfahrt and St. Peter	800	N 46°30′; E 11°22′
8	20030731-A1	I/TN/Ritten, between Forsthütte Lodn and Lengsteinplatz	1670	N 46°34′; E 11°28′
9	20030808-B1	A/SM/Rax, between Gasthof Moassa and Jahnhütte	1280	N 47°48′; E 15°45′
10	20030813-A2	A/KN, between Eisenkappel and Ebriach	670	N 46°28′; E 14°31′
11	20030813-B2	SLO/N Sv. Jakob, W ČErna	1120	N 46°29′; E 14°43′
12	20030814-A2	SLO/W Zali Log, between Zelezniki and Podbrdo	560	N 46°12′; E 14°05′
13	20030822-A1	D/NW/Aachen, Vennbahnweg	270	N 50°44′; E 06°09′
14	20030825-A1	D/NW, between Vennwegen and Kornelimünster	260	N 50°43′; E 06°11′
15	20030825-B1	B/LI, between Vennkreuz and Bellesforter Brücke	410	N 50°38′; E 06°07′
16	20030828-A1	D/RP, between BAB exit Emmelshausen and Karbach	500	N 50°09′; E 07°36′
17	20030828-B1	D/BW, Obersontheim	400	N 49°03′; E 09°52′
18	20030902-A1	D/ST, Schierke	630	N 51°46′; E 10°39′
19	20030907-A1	D/ST/Wernigerode, "Beerenstieg" below "Hohne Klippen"	760	N 51°46′; E 10°42′
20	20030909-A1	A/T/Innsbruck, Neue Universität	590	N 47°15′; E 11°23′
21	20030910-A1	D/ST/IPK-Gatersleben	115	N 51°49′; E 11°16′
22	20010204-A1	NZ/OT, Otago Coast Forest	130	S 46°08′; E 170°07′
23	20030717-A1	FIN/Uusimaa, Kirkkonummi	–	N 60°07′; E 24°27′[b]
24	20030717-A2	N/Akershus/Oslo, Lindoya	–	N 59°55′; E 10°46′[b]

[a] Also including information about collection date (YYYYMMDD).
[b] Grown at Botancal Garden Innsbruck (coordinates: N 47°16′; E 11°22′; altitude: 620 m a.m.s.l.) from seeds obtained from Botanical Gardens of Helsinky and Oslo, respectively.

Table 2
Absolute quantification results of flavonoids (**F1**–**F4**) and phenolic acids (**P1**–**P4**) (mg/g) in dried flowerheads of *Leontodon autumnalis* accessions 1–24 (SD = standard deviation) [a,b]

Accession	Total mg/g		**F1**		**F2**		**F3**		**F4**		**P1**		**P2**		**P3**		**P4**	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
1	16.88	0.28	1.75	0.03	4.33	0.11	1.00	0.04	3.37	0.07	1.67	0.09	1.01	0.03	0.65	0.02	3.10	0.09
2	22.29	0.23	2.93	0.07	2.94	0.04	1.35	0.08	3.47	0.16	2.44	0.10	2.38	0.05	0.95	0.02	5.83	0.10
3	19.32	0.17	2.83	0.02	2.43	0.04	1.36	0.14	4.05	0.21	1.70	0.10	1.89	0.01	0.81	0.04	4.23	0.10
4	27.35	0.99	1.44	0.02	5.92	0.31	1.32	0.07	4.46	0.03	3.76	0.22	2.37	0.12	1.08	0.06	7.02	0.22
5	19.07	0.05	1.16	0.00	4.08	0.04	1.49	0.01	3.56	0.13	2.86	0.01	1.22	0.02	0.77	0.00	3.94	0.01
6	18.90	0.10	2.43	0.04	3.33	0.02	1.09	0.00	3.56	0.17	2.13	0.01	1.25	0.02	0.84	0.00	4.27	0.01
7	18.95	0.02	1.70	0.00	3.62	0.02	1.14	0.01	3.22	0.04	1.58	0.07	1.73	0.01	0.82	0.01	5.14	0.07
8	16.23	0.24	2.32	0.05	2.81	0.03	0.48	0.00	2.98	0.02	1.49	0.08	1.31	0.03	0.69	0.01	4.15	0.08
9	24.60	0.51	1.61	0.00	3.89	0.15	1.60	0.09	3.13	0.07	3.58	0.23	2.69	0.08	1.19	0.01	6.92	0.23
10	19.26	0.50	1.86	0.04	4.20	0.11	1.19	0.06	3.60	0.07	1.88	0.10	2.03	0.03	0.66	0.02	3.85	0.10
11	17.66	0.43	2.72	0.12	3.28	0.06	1.09	0.06	2.50	0.03	1.93	0.08	1.84	0.02	0.62	0.02	3.67	0.08
12	18.67	0.44	1.60	0.09	3.32	0.07	1.51	0.05	2.66	0.03	2.12	0.10	1.86	0.00	0.92	0.03	4.68	0.10
13	22.94	0.01	2.23	0.01	3.67	0.01	1.28	0.02	3.66	0.00	2.65	0.03	2.38	0.00	1.07	0.00	5.99	0.03
14	17.31	0.25	1.41	0.02	3.30	0.08	1.44	0.05	3.05	0.07	1.65	0.06	1.49	0.05	0.69	0.00	4.28	0.06
15	20.11	0.01	2.36	0.01	3.41	0.03	1.19	0.10	3.59	0.01	1.91	0.05	2.08	0.05	0.75	0.01	4.84	0.05
16	19.00	0.00	1.36	0.00	3.22	0.02	1.41	0.10	2.72	0.03	2.55	0.02	1.59	0.05	0.90	0.01	5.26	0.02
17	19.29	0.20	1.72	0.00	4.25	0.02	1.88	0.14	3.65	0.04	1.87	0.01	1.76	0.06	0.63	0.01	3.54	0.01
18	23.06	0.13	1.65	0.03	4.43	0.04	1.72	0.14	3.96	0.06	2.19	0.03	1.86	0.09	0.91	0.01	6.33	0.03
19	29.10	1.48	2.15	0.06	5.11	0.20	1.63	0.23	4.81	0.16	3.05	0.71	2.48	0.02	1.09	0.04	8.77	0.71
20	19.11	0.46	2.04	0.05	3.51	0.10	1.38	0.05	2.44	0.04	1.75	0.18	1.88	0.08	1.00	0.02	5.12	0.18
21	20.18	0.02	1.85	0.02	3.22	0.02	1.32	0.11	2.62	0.02	1.98	0.03	1.91	0.05	1.09	0.00	6.20	0.03
22	23.42	0.22	3.30	0.01	3.85	0.03	1.44	0.13	3.95	0.01	1.95	0.07	2.40	0.04	0.95	0.01	5.57	0.07
23	19.83	0.30	2.00	0.05	2.51	0.09	1.16	0.02	4.15	0.14	1.73	0.17	1.23	0.02	1.13	0.04	5.91	0.17
24	18.98	0.22	2.02	0.01	3.05	0.01	1.47	0.07	4.32	0.17	1.65	0.04	1.30	0.04	0.75	0.00	4.42	0.04

[a] Results of HPLC/DAD investigations; amounts estimated by ratio of peak areas to area of quercetin as internal standard.
[b] Flavonoids: **F1** = luteolin, **F2** = luteolin 7-*O*-β-D-glucoside, **F3** = luteolin 7-*O*-β-D-glucuronide, **F4** = luteolin 4′-*O*-β-D-glucoside. Phenolic acids: **P1** = chlorogenic acid, **P2** = 3,5-dicaffeoyl quinic acid, **P3** = caffeoyl tartaric acid, **P4** = cichoric acid.

Table 3
Absolute quantification results of sesquiterpenoids (**S1**–**S6**) (ppm) in dried subaerial parts of *Leontodon autumnalis* accessions 1–24 (SD = standard deviation)[a,b]

Accession	$\sum_{S1-S6}$ ppm		**S1**		**S2**		**S3**		**S4**		**S5**		**S6**	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
1	2461	46	1824	51	437	5	n.d.	–	n.d.	–	(+)	–	(+)	–
2	3029	37	285	6	208	5	1327	12	401	8	406	4	400	11
3	1396	99	904	101	292	2	n.d.	–	n.d.	–	(+)	–	(+)	–
4	4341	58	3101	38	325	1	(+)	–	n.d.	–	(+)	–	715	22
5	3302	47	2219	50	452	2	(+)	–	n.d.	–	(+)	–	430	5
6	2345	40	1352	32	447	2	(+)	–	n.d.	–	(+)	–	346	10
7	4559	335	3416	309	533	2	(+)	–	n.d.	–	(+)	–	410	29
8	3075	31	2287	30	353	6	(+)	–	n.d.	–	(+)	–	235	7
9	5932	34	4824	27	383	1	(+)	–	n.d.	–	(+)	–	524	7
10	3445	199	2431	179	404	5	(+)	–	(+)	–	(+)	–	310	25
11	3382	56	2294	39	313	3	(+)	–	n.d.	–	(+)	–	575	21
12	5209	130	4028	125	593	4	(+)	–	(+)	–	(+)	–	288	9
13	3831	55	956	33	(+)	–	1647	28	397	6	490	7	240	7
14	2970	26	707	18	(+)	–	999	7	317	5	406	5	441	10
15	1174	23	(+)	–	(+)	–	439	21	(+)	–	335	2	(+)	–
16	2726	63	1861	69	565	6	(+)	–	n.d.	–	(+)	–	(+)	–
17	4968	77	1344	45	573	3	1868	38	562	6	302	8	318	12
18	2423	49	402	18	(+)	–	1086	24	301	4	244	3	290	13
19	6797	65	5874	64	486	2	(+)	–	n.d.	–	(+)	–	237	4
20	3182	96	2156	84	392	5	(+)	–	(+)	–	(+)	–	334	17
21	4737	111	3159	101	742	10	(+)	–	(+)	–	(+)	–	535	20
22	2958	19	2347	19	311	1	(+)	–	n.d.	–	(+)	–	(+)	–
23	3573	100	2458	86	515	3	(+)	–	n.d.	–	(+)	–	401	10
24	4384	31	1215	20	(+)	–	2544	12	(+)	–	224	1	202	2

[a] Results of HPLC/DAD investigations; amounts estimated by ratio of peak areas to area of santonin as internal standard; n.d. = not detectable, (+) = traces, i.e., <200 ppm.

[b] Sesquiterpenoids: **S1** = crepidiaside A, **S2** = crepidiaside B, **S3** = 15-glucopyranosyloxy-2-oxo-guaia-3,11(13)-dien-1α,5α,6β,7α,10αH-12,6-olide, **S4** = 15-glucopyranosyloxy-2-oxo-guaia-3-en-1α,5α,6β,7α,10α,11βH-12,6-olide, **S5** = 15-hydroxy-2-oxo-guaia-3-en-1α,5α,6β,7α,10α,11βH-12,6-olide, **S6** = 15-glucopyranosyloxy-2-oxo-guaia-3,11(13)-dien-1β,5α,6β,7α,10αH-12,6-olide.

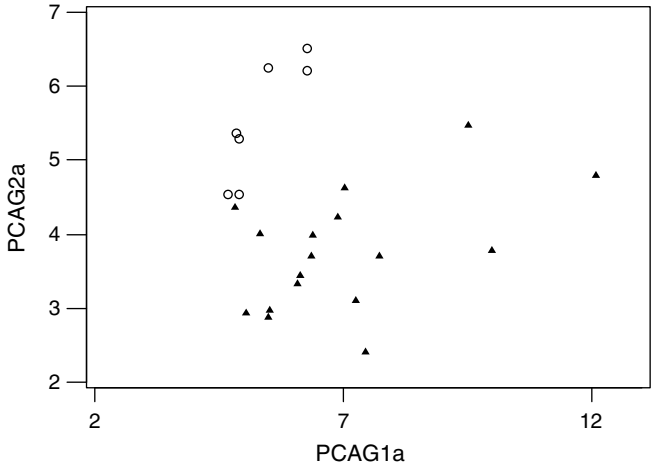

Fig. 3. Scatter plot of the scores for the first versus the second principal component obtained from the absolute quantification data matrix of flavonoids, phenolic acids, and sesquiterpenoids.

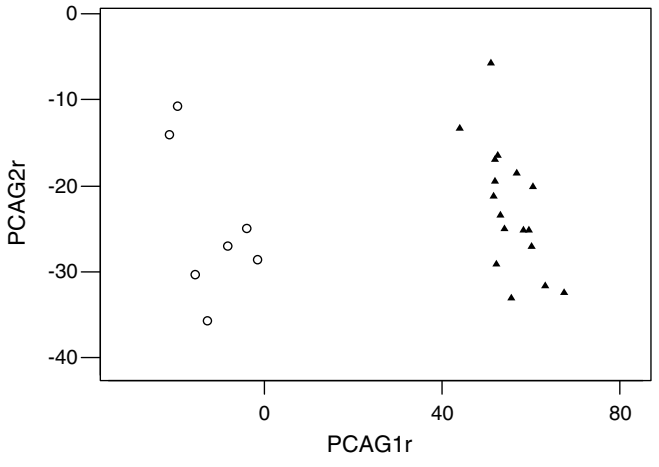

Fig. 4. Scatter plot of the scores for the first versus the second principal component obtained from the relative quantification data matrix of flavonoids, phenolic acids, and sesquiterpenoids.

4). A total of 183 individual plants (four to eight per population) were analyzed and the investigation resulted in 77 clearly reproducible molecular marker fragments. An example genetic profile obtained with primer C12 and individuals from populations 11, 12, and 13 is shown in Fig. 7. Observed RAPD bands were coded either due to their presence/absence (1,0) or absence/presence and intensity (0,1,2,3). The resulting unrooted trees calculated with PAUP* 4.0 via neighbor-joining (NJ) analyses of both data sets were very similar, which is the reason

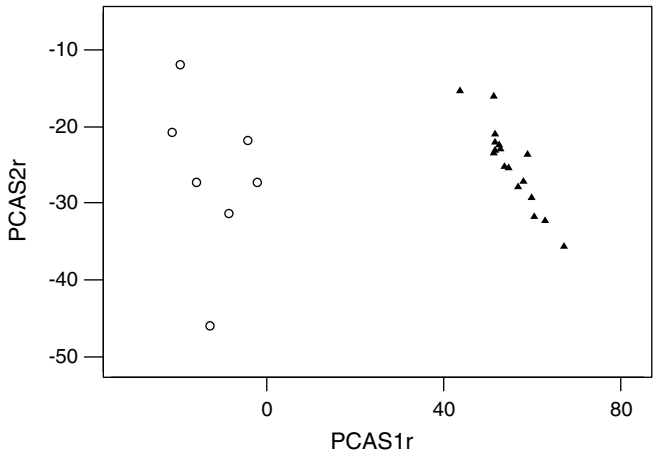

Fig. 5. Scatter plot of the scores for the first versus the second principal component obtained from the relative quantification data matrix of sesquiterpenoids.

why we show only the tree derived from the 0/1/2/3-data matrix (Fig. 8). Individuals belonging to one accession usually grouped together. Monophyletic groups are composed of one to four different populations. Bootstrap values, indicating the statistical support of the branches, were low throughout the tree ($\leqslant$76%) and therefore not shown in Fig. 8.

3. Discussion

The existence of two distinct chemotypes in *L. autumnalis*, which were discovered in earlier investigations (Zidorn et al., 2000; Zidorn and Stuppner, 2001a) was confirmed. The strict geographic separation of these chemotypes, which was implied by previous results, was however not confirmed. While chemotype B seems to be restricted to areas North and Northwest of the Alps, populations of chemotype A are occurring in the Alps, Central Europe, England, and Finland. At least in Southern England and Central Germany both chemotypes co-occur.

The clusters derived from RAPD analysis (Fig. 8) that consist of more than one accession were mostly comprised of populations, which are geographically close to each other (4, 5, and 6; 1 and 7; 13, 14, and 15). However, three geographically remote populations (12, 16, and 23) group together. Despite of some misplaced individuals (18a, 18b, and 21f) the recognized clusters comprised only populations from the same chemotype. Although four of the seven populations from chemotype B (13, 14, 15, and 17) form a monophyletic group, genetic profiles did not correlate with the chemotypes. The situation is further complicated by the fact that chemically and geographically uniform populations from the Aosta Valley (4, 5, and 6) show a high degree of morphological variation. Population

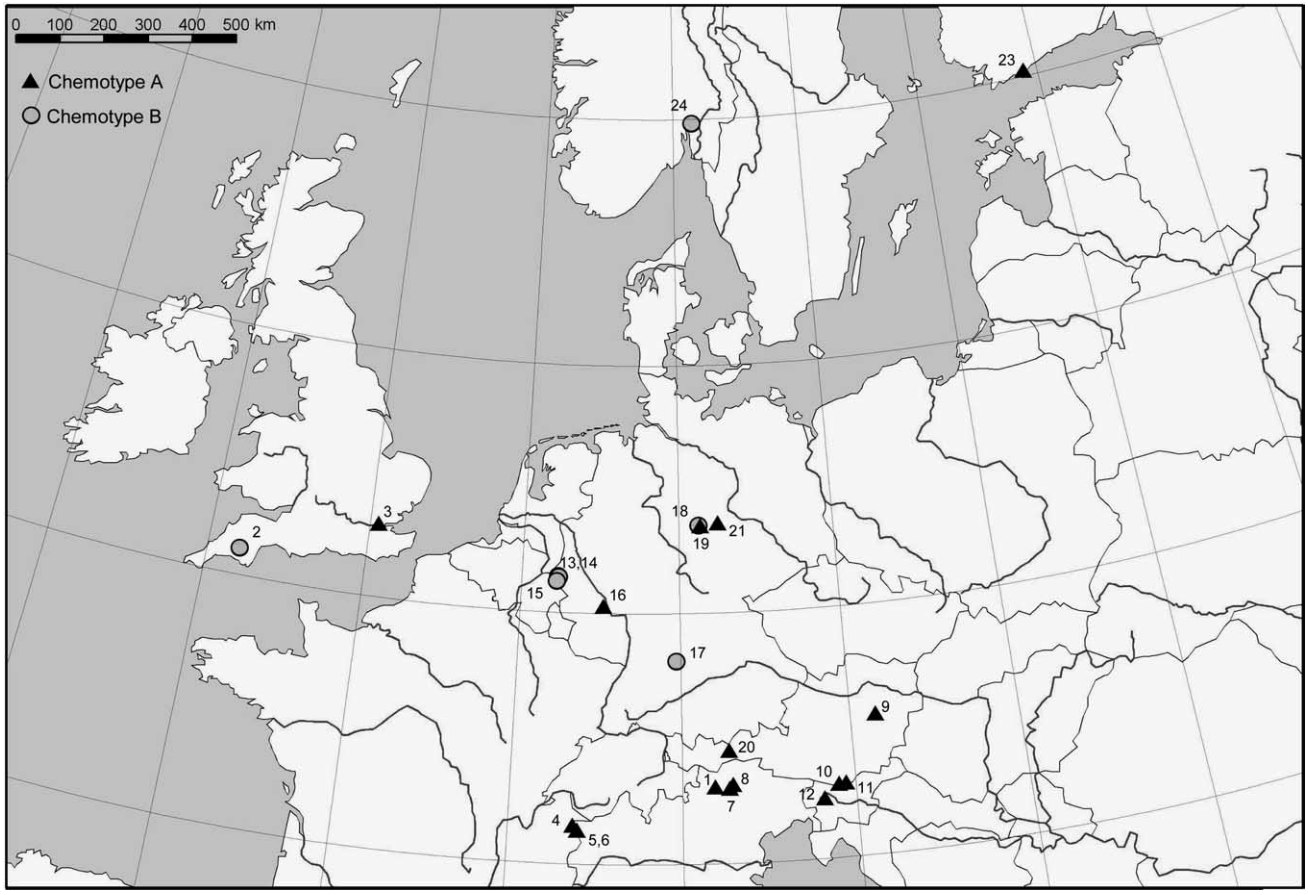

Fig. 6. Distribution of *Leontodon autumnalis* chemotypes. The numbers refer to the accession numbers given in Table 1.

Table 4
Primers employed and the number of RAPD markers obtained

Primer	Sequence (5′ → 3′)	Size (bp) min–max	Number of bands		
			Polymorphic	Monomorphic	Total
A19	CAAACGTCGG	760–380	2	2	4
AB04	GGCACGCGTT	1000–450	2	2	4
AC2	GTCGTCGTCT	1030–380	3	3	6
B08	GTCCACACGG	1020–540	2	2	4
B10	CTGCTGGGAC	920–530	5	1	6
B11	GTAGACCCGT	920–500	3	0	3
B12	CCTTGACGCA	500–290	2	1	3
B15	GGAGGGTGTT	870–280	6	2	8
B18	CCACAGCAGT	760–300	3	2	5
C07	GTCCCGACGA	530–350	2	1	3
C12	TGTCATCCCC	1450–260	7	2	9
D02	GGACCCAACC	1200–550	2	2	4
E01	CCCAAGGTCC	1100–490	3	2	5
E19	ACGGCGTATG	970–440	3	1	4
G14	GGATGAGACC	580–170	2	2	4
G19	GTCAGGGCAA	820–360	2	3	5

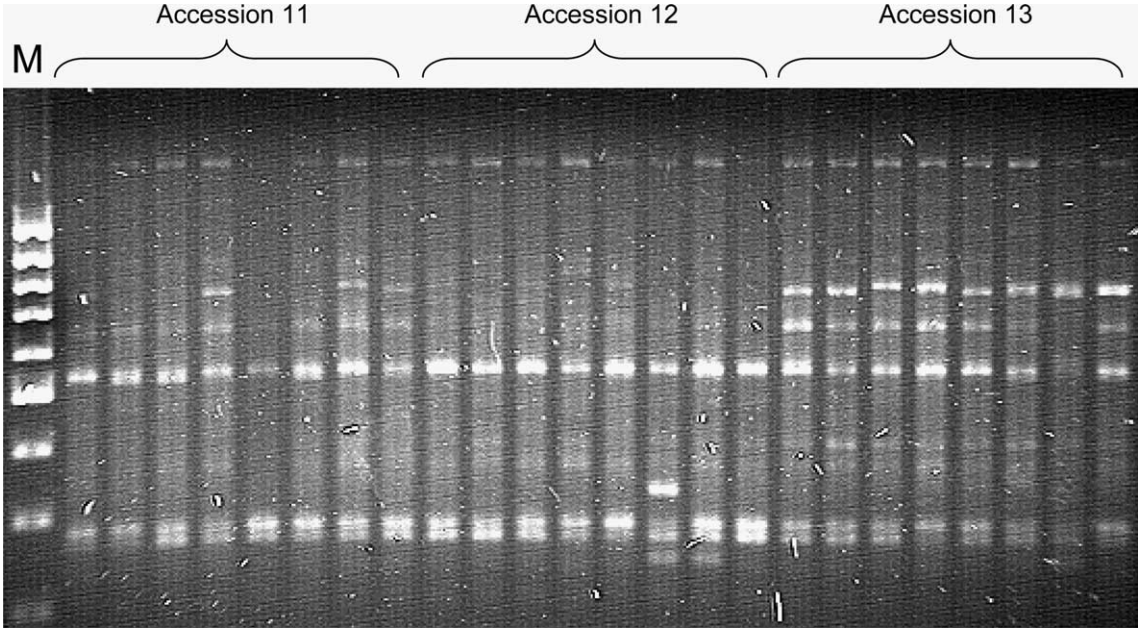

Fig. 7. Example of an ethidium bromide stained agarose gel of a RAPD reaction of Leontodon *autumnalis* accessions 11, 12, and 13 with Operon primer C12; M = size standard (100 bp ladder).

4 comprised individuals with few small capitula without a pronounced indumentum of the involucral bracts; population 5 consisted of individuals with only one small capitulum per scape, which also lacked a pronounced indumentum; and population 6 was comprised of individuals with few relatively large capitula with a pronounced grey involucrum (similar to *Leontodon montanus* Lam.). Conclusively, populations 4 and 5 are referable to *L. autumnalis* L. subsp. *autumnalis* sensu Flora Europaea (Finch and Sell, 1976) and population 6 shares some characteristics of *L. autumnalis* L. subsp. *pratensis* (Koch) Arcangeli.

As different accessions from Scandinavia grown under identical conditions in the Innsbruck Botanic Gardens were assigned to different chemotypes, a genetical basis for the observed chemical differences has to be assumed and it is less likely that chemical differences are merely resulting from reactions of the plants/populations in response to, e.g., microbial attacks (phytoalexins).

The following conclusions can be drawn from the presented data. (1) More investigations are needed to clearly define the geographical areas of the two detected chemotypes. (2) Morphological variability within the *L. autumnalis* group is at least partially due to a great plasticity of the taxon. Intraspecific entities described by other authors have to be re-evaluated with great care with regards to their taxonomic significance. (3) Although chemical differences have most likely a genetic foundation,

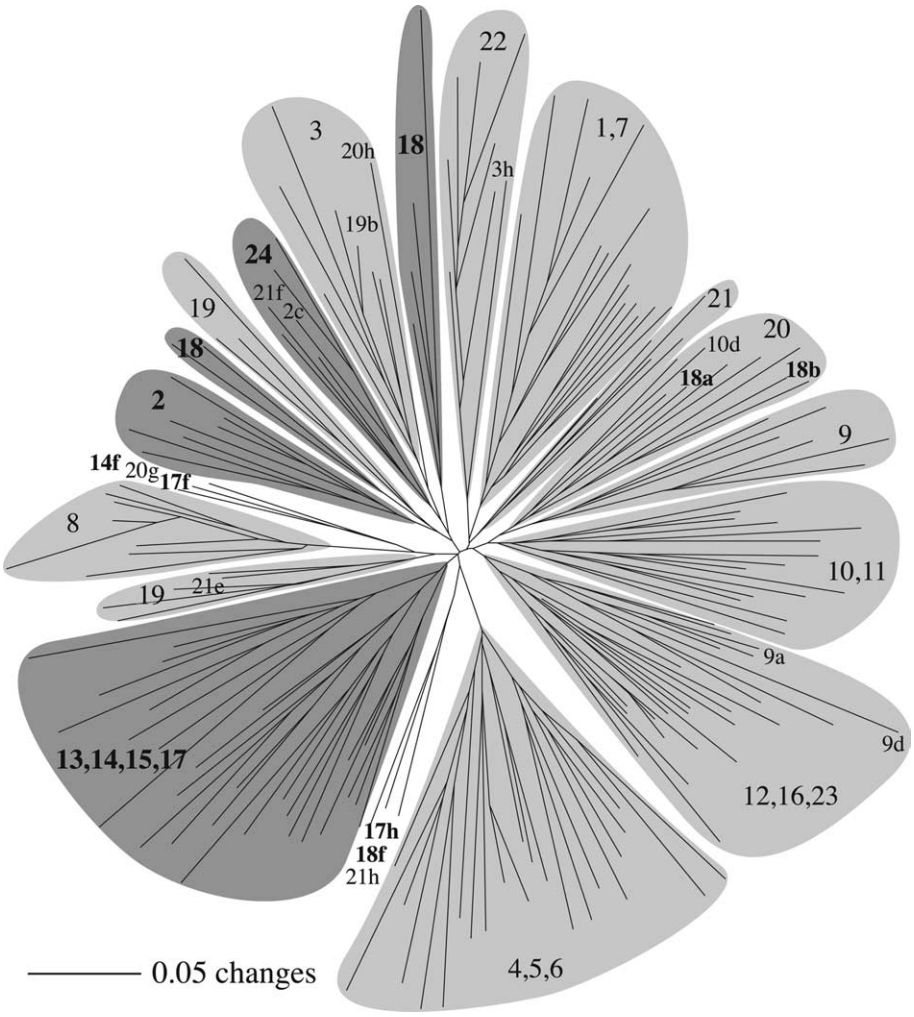

Fig. 8. Unrooted tree from the neighbor-joining analysis based on the 0/1/2/3-data matrix of 183 individuals and 77 RAPD markers. Monophyletic groups consisting of individuals from specific populations were marked by gray areas, while misplaced individuals or individuals without clear group association were depicted by the population number (Table 1) and the extension a–h. Dark gray shading and bold population numbers indicate chemotype B members, whereas light shading and roman numbers indicate chemotype A.

genetic differences between populations of both chemotypes are not pronounced. Therefore, small differences in the genome might be responsible for the observed phytochemical differences. Phytochemical variation requires in some instances the mutation of a few genes only (Crawford, 1978). Therefore, multiple origins of the observed chemotypes cannot be ruled out. (4) The ecological significance of the detected phytochemical variation within *L. autumnalis* remains unknown.

4. Experimental

4.1. Plant material

A total of 24 accessions of *L. autumnalis* were collected in Central Europe, from the Alps (Austria, Italy, and Slovenia) to Northwestern Central Europe (Germany and Belgium), Great Britain, and to Northern Europe (Norway and Finland). Additionally, one population from New Zealand and two grown from seeds obtained from Scandinavia (Norway and Finland) were analyzed (Table 1). For RAPD experiments each accession was represented by 4–8 individuals, giving a total of 183 variables. Voucher specimens of all investigated populations are preserved in the Herbarium of the Institut für Pharmazie, Universität Innsbruck, Austria. Voucher codes are included into Table 1.

4.2. Abbreviations of political districts and states

A = Austria, AO = Aosta, B = Belgium, BW = Baden-Württemberg, D = Germany, FIN = Finland, GB = Great Britain, I = Italy, KN = Carinthia, LI = Liége, N = Norway, NW = North Rhine-Westphalia, NZ = New Zealand, OT = Otago, RP = Rhineland-Palatinate, SLO = Slovakia, SM = Styria, ST = Saxony-Anhalt, T = Tyrol, TN = Trentino/South Tyrol.

4.3. Phytochemistry data collection

4.3.1. Sample preparation

Flavonoids and phenolic acids were extracted using the protocol described by Zidorn et al. (2005).

Sesquiterpenoids were extracted from subaerial parts using a modification of the method described by Zidorn et al. (2000). In the present study Ultraturrax extraction cycles were replaced by sonication (3 times for 30 min).

4.3.2. HPLC analysis

Flavonoids (**F1** = luteolin, **F2** = luteolin 7-*O*-β-D-glucoside, **F3** = luteolin 7-*O*-β-D-glucuronide, and **F4** = luteolin 4′-*O*-β-sc d-glucoside) and phenolic acids (**P1** = chlorogenic acid, **P2** = 3,5-dicaffeoyl quinic acid, **P3** = caffeoyl tartaric acid, and **P4** = cichoric acid) were identified and quantified as described previously (Zidorn and Stuppner, 2001a) (see also Fig. 2b). Table 2 shows mean values and standard deviations of absolute quantification results of phenolic compounds.

Sesquiterpenoids (**S1** crepidiaside A, **S2** crepidiaside B, **S3** 15-glucopyranosyloxy-2-oxo-guaia-3,11(13)-dien-1α,5α,6β,7α,10αH-12,6-olide, **S4** 15-glucopyranosyloxy-2-oxo-guaia-3-en-1α,5α,6β,7α,10α,11βH-12,6-olide, **S5** 15-hydroxy-2-oxo-guaia-3-en-1α,5α,6β,7α,10α,11βH-12,6-olide, **S6** 15-glucopyranosyloxy-2-oxo-guaia-3,11(13)-dien-1β,5α,6β,7α,10αH-12,6-olide) were identified and quantified using a modification of the system described by Zidorn et al. (2000). In detail, column, Zorbax SB-C18, 4.6 × 150 mm, particle size 3.5 μm; guard column, LiChroCART 4 × 4 mm packed with LiChrospher 100 RP-18 material (5 μm particle size); mobile phase A, H_2O; mobile phase B, MeCN; linear gradient from 12% B to 15% B in 15 min, 35% B in 10 min, 60% B in 5 min, and 98% B in another 5 min stop time 45 min, post time 12 min; flow rate 1.00 ml/min; detection wavelength: 245 nm; injection volume: 10 μl; oven temperature, 40 °C. The following retention times were observed: **S1**, 18.9 min; **S2**, 20.1 min; **S3**, 15.1 min; **S4**, 17.6 min; **S5** 21.5 min; **S6**, 22.0 min; santonin (internal standard), 28.4 min (see also Fig. 2a). All quantifications were performed in triplicate. Contents of sesquiterpenoids were estimated by ratio of peak areas to area of santonin as internal standard. Mean values and standard deviations of absolute quantification results of sesquiterpenoids are given in Table 3.

4.3.3. Phytochemical data evaluation

Principal component analyses (PCA) were performed with the Minitab statistical software package 13.31 (Minitab, State College, USA). PCAs were performed from the covariance data matrix.

4.4. RAPD data collection

4.4.1. Isolation of DNA from leaf tissue

Genomic DNA (i.e., total DNA) was extracted using a modification of the Qiagen DNeasy Plant Mini Kit™ protocol (Qiagen GmbH, Hilden, Germany) as described by Blattner and Kadereit (1999). The quality and concentration of the extracted DNA was checked on an agarose gel. To obtain approximately equal DNA concentrations, the DNA solutions were – if necessary – diluted with water. Ten microliter of the isolated DNA solution were dissolved in 100 μl of water and 1 μl of this DNA solution was used in RAPD amplifications.

4.4.2. RAPD amplification

PCR was performed in a 10 μl reaction volume, containing 1–3 ng of target DNA, 50 pM random decamer primer (Operon Technologies, Alameda, CA), 875 pM dNTPs (Roth & Co.KG, Karlsruhe, Germany), 0.2 units of thermostable *Taq* DNA polymerase, 1 μl PCR puffer solution (10×), 0.5 μl $MgCl_2$ solution (25 mM), and 2 μl Q-solution (5×) (all from Qiagen GmbH, Hilden, Germany). Amplifications were carried out in a GeneAmp PCR System 9700 Thermal Cycler (Perkin–Elmer, Boston, USA) programmed for an initial 3 min denaturating at 94 °C, followed by 35 cycles of 0.5 min annealing at 43 °C, 2 min extension at 72 °C, and 0.5 min denaturation at 95 °C, with a posttreatment of 10 min at 72 °C. Prior to the analysis of the entire set of accessions, about 50 RAPD primers were tested on a small subset of plants. Final amplifications were carried out using 16 arbitrary decamer primers (Table 4), that provided clear and reproducible bands in the initial screening. The RAPD reaction mixtures were separated on 1.5% agarose gels in 0.5× TBE buffer and stained with ethidium bromide. Only clearly visible RAPD bands (an example is given in Fig. 7) were scored manually either for presence (1) or absence (0) or band intensity was taken into account with absence (0) and presence coded on a scale from 1 to 3. Band scoring was done using enlarged photographs of the gels.

From the resulting data matrices neighbor-joining analyses were calculated with PAUP* 4.0 (Swofford, 2002) using either Nei and Li genetic distances (0/1-matrix) or mean character differences (0/1/2/3-matrix). Statistical support of the branches was evaluated with 500 bootstrap resamples.

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