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Glucosinolates in *Diplotaxis* and *Eruca* leaves: Diversity, taxonomic relations and applied aspects

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Abstract

Leaf glucosinolates of 42 *Diplotaxis* and 21 *Eruca* accessions were studied. Total content ranged from 0.25 to more than 70 g kg^{-1} dry wt. The 13 clusters, defined on the basis of glucosinolate composition, belonged to two glucosinolate-rich groups, characterised by the prevalence of a single component, and one low-glucosinolate group, with a profile not dominated by any individual component. A sinigrin-rich cluster (*D. ibicensis*, *D. berthautii*, *D. ilorcitana*, *D. siettiana*, *D. tenuisiliqua*, *D. brevisiliqua*, and *D. virgata*) and a gluconapin-rich cluster (*D. catholica*, *D.siifolia*, *D. virgata*, and *D. ollivieri*) included all the species previously classified in the *nigra* phylogenetic lineage. *D. virgata* was confirmed to be a critical taxon, with one accession slightly diverging from the others. *D. siifolia* subsp. *vicentina* was separated from the others in a glucobrassicin-rich cluster. *D. harra*, a rather isolated representative of sub-genus *Hesperidium*, clustered together *D. assurgens* in a sinalbin-rich cluster. Another well defined cluster was represented by *D. brachycarpa* (gluconasturtin). The two sub-species of *D. erucoides* were well differentiated by their glucosinolate profile. The low glucosinolate species: *D. tenuifolia*, *D. viminea*, *D. cretacea*, *D. muralis* (subgenus *Diplotaxis*), and *E. vesicaria*, all previously included in the *rapa/oleracea* lineage, belonged to seven less defined clusters, mainly differing on the presence/absence or the relative abundance of some components (glucoraphanin, glucolepidin, 4-hydroxy-glucobrassicin, 4-phenylbutyl gls, glucoerucin and neoglucobrassicin). The data support previous taxonomic works. Glucosinolate-rich taxa, with well characterised profiles may be suitable for industrial uses, whereas the variability of edible *D. tenuifolia* and *E. vesicaria* may represent a basis for breeding horticultural types.
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1. Introduction

The genera *Diplotaxis* and *Eruca* (family Brassicaceae), exhibit most of the primitive morphological characters of the tribe Brassicinae (Martinez-Laborde, 1997). *Diplotaxis* and *Eruca* are phylogenetically close to the economically important genus *Brassica* (Harberd, 1972), and have been recognised as potential sources of useful characters for breeding cultivated brassicas (Sharma et al., 2002).

The genus *Diplotaxis* DC is native to the Mediterranean region. The genus, with more than 30 species, is currently distributed from Central Europe to India and West Africa, and naturalised in the New World (Martinez-Laborde, 1997). The taxa have been taxonomically investigated by means of morphological characters (Martinez-Laborde, 1991; Gomez-Campo and Martinez-Laborde, 1998), biochemical components such as flavonoids (Sanchez-Yelamo and Martinez-Laborde, 1991; Sanchez-Yelamo, 1994), seed proteins (Sanchez-Yelamo et al., 1992), isoenzymes (Sanchez-Yelamo and Martinez-Laborde, 1991), and molecular markers (Pradhan et al., 1992; Warwick et al., 1992; Martin and Sanchez-Yelamo, 2000; Eschmann-Grupe et al., 2003; Warwick and Sauder, 2005). These contributions represent

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the basis for periodic reviews of the taxonomic classification, and the investigation of phylogenetic relationships at the generic, sub-generic, specific and sub-specific levels (Martinez-Laborde, 1997; Gomez-Campo, 1999; Al-Shehbaz et al., 2006).

Eruca vesicaria (L.) Cav. grows wild in the Mediterranean region, but its primary area of origin is not well defined, having been extensively exploited as a vegetable or oilseed in several parts of the world. The genus is monospecific, with three subspecies described (Gomez-Campo, 1999), sometimes elevated at the rank of independent species. All taxa have been characterised by means of morphological traits, and some accessions were included in some of the cited taxonomic investigations. This species was also used as a model for glucosinolate biosynthesis investigations (Falk et al., 2004).

Diplotaxis tenuifolia (L.) DC and *E. vesicaria* subsp. *sativa* (Miller) Thell. are important edible species: *D. tenuifolia*, and the pungent forms of *E. vesicaria* subsp. *sativa*, represent, respectively, the "wild" and "cultivated" rocket salads, whereas non pungent forms of *Eruca* are popular vegetables in the Middle East.

The whole order Brassicales (APG II, 2003), (formerly Capparales, Cronquist, 1988) is characterised by the presence of glucosinolates, the nature and function of which have been thoroughly illustrated (Kjaer, 1976; Stoewsand, 1995; Rosa et al., 1997; Fahey et al., 2001; Halkier and Gershenzon, 2006), with special respect to adverse (Heaney and Fenwick, 1985, 1995) and positive effects on human and animal health, (Nestle, 1998; Srinibas et al., 2000), their potential for the control of some soil borne diseases and pests (Kirkegaard et al., 1998), and their impact on flavour and acceptance of feeds or human foods (Rosa, 1999; Drewnowski and Gomez-Carneros, 2000).

Although many edible and industrial species have been characterised for glucosinolate content and composition, recently also including *Diplotaxis* and *Eruca* (Bennett et al., 2006), glucosinolates have rarely been considered as biochemical markers for taxonomic investigations.

The main aim of this work was the evaluation of glucosinolate content and composition of several *Diplotaxis* and *Eruca* gene bank accessions to review their potential for exploitation as fresh vegetables, nutraceutical raw materials, or biomass for the control of soil borne pests and diseases in short-term intensive rotations (biofumigation). For these reasons, only mature leaves were considered in this paper. An attempt was made to relate our results to the results of previously cited taxonomic investigations. Finally, some applied aspects are briefly discussed.

2. Results

At harvest the plants of all accessions were at the same developmental stage. Apart from some differences in plant population size, all the accessions were homogeneous and did not suffer pests or diseases. These were prerequisites in order to minimise glucosinolate variation due to ontogenetic or environmental factors (Bennett et al., 2006).

The 20 glucosinolates identified and their identification details are shown in Table 2. The last compound (g21) corresponds to the dimeric form of 4-mercaptobutyl glucosinolate (4-MER), reported as the major component of *E. vesicaria*, and also found in *D. erucoides* and *D. tenuifolia* (Bennett et al., 2006). This compound was isolated by a specific procedure (Bennett et al., 2002), and also detected in substantial amounts by Kim et al. (2006) using a standard extraction protocol. However, this was not our experience, where presence of the dimer was erratic: in consequence, 4-MER was not included in further data processing.

The total glucosinolate content, the relative content of the identified components, and non-hierarchical cluster membership of each accession are shown in Table 3.

Total glucosinolates ranged from values equivalent to those occurring in other major vegetables, for *E. vesicaria* and *D. tenuifolia*, to the very high amounts in *D. harra* and *D. siifolia*, more commonly found in seeds.

Three well defined groups were defined by some basic quantitative glucosinolate traits (Fig. 1). Group A: low total glucosinolate content, and profiles not dominated by any specific components (maximum relative abundance <60%), included all the edible *D. tenuifolia* and *E. vesicaria* accessions. Group B: medium or high total glucosinolate content, and strongly characterised profile (relative content of the more abundant component 80–100%). Group C: high or very high total glucosinolates, fairly well characterised profile, and a positive relation between glucosinolate content and the relative content of the main component.

Within-group variability was present for both glucosinolate presence and relative amounts. In fact, groups were further split into 13 clusters by means of non-hierarchical clustering, a result corresponding to 100% correct classification, with the exception of a single replication of *D. harra* a12, and no further significant reduction of the overall between-cluster variance. Fig. 2 represents the discriminant factor cluster profiles. The correlations between the discriminant factors and the retained glucosinolates from the backward selection procedure are shown in Table 4.

Clusters c3 and c4 derive from the splitting of group B. Cluster c3, including *D. ibicensis*, *D. berthautii*, *D. ilorcitana*, *D. siettiana*, *D. tenuisiliqua*, *D. brevisiliqua*, *D. virgata* a32, *D. ollivieri* a381, and *D. erucoides* a401, was well defined by discriminant factor df1 (Fig. 2), negatively correlated to sinigrin content. Cluster c4 (*D. catholica*, *D. siifolia* a20 and a22, *D. virgata* a31, *D. ollivieri* a382) was characterised by discriminant factor df2 (Fig. 2), negatively correlated to the gluconapin content.

Two single-accession, group A, clusters had well characterised glucosinolate profiles. Cluster c8 (*D. brachycarpa*) was discriminated by factor df3, because of its 46.6% gluconasturtin. It was, however, well defined also by factor df2, due to its 47.5% gluconapin, and the absence of glucoraphanin. Cluster c7 (*D. erucoides* a402) had a unique

Table 1
List of the considered taxa and accessions

Acc.[a]	Taxon (botanical name)[b]	Donor[c]	Collection site[d]	Accession number or name[d]
a141,a142	*Diplotaxis assurgens* (Del.) Gren.	DBM	Beni Mellal, E. Marrakech, Morocco	254-1120-67
a15	*Diplotaxis berthautii* Br.-Bl. & Maire	DBM	Jbilet region, N. Marrakech, Morocco	255-1079-67
a23	*Diplotaxis brachycarpa* Godr.	DBM	Roadsides, N. Sidi Aissa, Algeria.	256-6467-84
a39	*Diplotaxis brevisiliqua* (Coss.) Mart.-Lab.	DBM	Cala Iris, Rif coast, N. Morocco	257-7517-86
a25	*Diplotaxis catholica* (L.) DC.	DBM	Sandy soils, N. Madrid city, C. Spain	258-1390-68
a401,a402	*Diplotaxis erucoides* (L.) DC.	DBM	As a weed in Albarracín, Teruel, E. Spain	260-1235-67
a27	*Diplotaxis harra* (Forsk.) Boiss.	DBM	S. of Ain Sefra, W. Algeria	263-1939-71
a29	*Diplotaxis harra* Boiss. subsp. *crassif*olia (Raf.) Maire	DBM	Coll. by Hendricksen in Sicily, Italy	264-5966-81
a12	*Diplotaxis harra* Boiss. subsp. *lagascana* (DC.) O. Bolós and J. Vigo	DBM	Cuevas, Almería, S.E. Spain	265-0913-66
a28	*Diplotaxis harra* Boiss. subsp. *confusa* Mart.-Lab.	DBM	Arid slopes, M'Chedallah, Alger, Algeria	266-1831-70
a13	*Diplotaxis ibicensis* (Pau) Gómez-Campo	DBM	Coll. by J.Y. Lesouef, N. Ibiza Isl., Spain	267-3457-76
a18	*Diplotaxis ilorcitana* (Sennen) Aedo & Mart.-Lab.	DBM	Tabernas, Almería, S.E. Spain	268-4065-76
a16,a17	*Diplotaxis muralis* (L.) DC.	DBM	B.G. Berlin-Dahlem, Germany	269-0990-68
a381,a382	*Diplotaxis ollivieri* Maire	DBM	S. de Goulumine, S.W. Morocco	270-9250-96
a19	*Diplotaxis siettiana* Maire	DBM	Alborán Island, S. Spain	271-3025-74
a20	*Diplotaxis siifolia* G.Kunze	DBM	Maritime sands, Sanlúcar, Cádiz, S.Spain	272-1447-68
a22	*Diplotaxis siifolia* subsp. *bipinnatifida* (Coss.) Mart.-Lab.	DBM	Sandy soil near Agadir, S. Morocco	273-2970-74
a21	*Diplotaxis siifolia* subsp. *vicentina* (P. Cout.) Mart.-Lab.	DBM	Sands, Cabo San Vicente, S. Portugal	274-7621-88
a30	*Diplotaxis simplex* (Viv.) Sprengel	DBM	Sands S. of Ain Sefra, W. Algeria	275-1931-71
a51	*Diplotaxis* sp.	IGB	Lecce, Italy	RN17
a45	*Diplotaxis* sp.	IGB	Lecce, Italy	RN18
a46	*Diplotaxis tenuifolia* (L.) DC	IGB	Lecce, Frigole (via Lopara,1) Italy	RN01
a47	*Diplotaxis tenuifolia* (L.) DC	IGB	Road Lecce-Torre Chianca, Italy	RN03
a48	*Diplotaxis tenuifolia* (L.) DC	IGB	Road Lecce-S. Cataldo – Loc. Marangi, Italy	RN04
a49	*Diplotaxis tenuifolia* (L.) DC	IGB	Road Cesine-S. Cataldo, Italy	RN06
a50	*Diplotaxis tenuifolia* (L.) DC	IGB	Road Lecce-S. Cataldo – Loc. Marangi, Italy	RN09
a67	*Diplotaxis tenuifolia* (L.) DC	IGB	Bratislava Revova street in garden	RN34
a52	*Diplotaxis tenuifolia* (L.) DC	SSC	Normandie, France	MT S.Michel
a53	*Diplotaxis tenuifolia* (L.) DC	SSC	Normandie, France	Sword Beach
a54	*Diplotaxis tenuifolia* (L.) DC	SSC	Unknown	Carnac
a55	*Diplotaxis tenuifolia* (L.) DC	SSC	Local selection, Cesena	Rucola selvatica
a56	*Diplotaxis tenuifolia* (L.) DC	SSC	Local selection, Cesena	Rucola selvatica sel. Liscia
a57	*Diplotaxis tenuifolia* (L.) DC	SSC	Local selection, Cesena	Selvatica a foglia frastagliata
a58	*Diplotaxis tenuifolia* (L.) DC	SSC	Local selection, Cesena	Selvatica a foglia d'ulivo
a36	*Diplotaxis tenuifolia* (L.) DC subsp. *cretacica* (Kotov) Sobrino-Vesperinas	DBM	B.G. Moscow, Russia	259-4189-76
a34	*Diplotaxis tenuifolia* (L.) DC.	DBM	Waste places near Istanbul, Turkey	276-0980-66
a35	*Diplotaxis tenuifolia* (L.) DC. f. *integrifolia*	DBM	Castle of Alarcón, Cuenca, E. Spain	277-5447-79
a24	*Diplotaxis tenuisiliqua* Del.	DBM	Roadsides near Tadla, C. Morocco	279-1123-67
a37	*Diplotaxis viminea* (L.) DC.	DBM	B.G. Munich, Germany	280-2108-76
a31	*Diplotaxis virgata* (Cav.) DC.	DBM	Sandy soil N. of Madrid city, C. Spain	281-0952-66
a32	*Diplotaxis virgata* (Cav.) DC. subsp. *australis* Mart.-Lab.	DBM	Arid pasturelands, N. Tazenakh, S. Morocco	282-3003-74
a26	*Diplotaxis virgata* (Cav.) DC. subsp. *rivulorum* (Br.Bl. & Maire) Mart.-Lab.	DBM	Marrakech, Morocco	283-3644-75
a43	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell	DBM	Waste fields near Marnia, N.W. Algeria	300-1796-70
a44	*Eruca vesicaria* (L.) Cav.	DBM	Gypsaceous soil near Seseña, S. Madrid, Spain	301-9101-95
a41	*Eruca vesicaria* (L.) Cav. subsp. *pinnatifida* (Desf.) Emb. & Maire	DBM	Between Djelfa and Bou Saada, Algeria	297-1813-70
a42	*Eruca vesicaria* (L.) Cav. subsp. *pinnatifida* (Desf.) Emb. & Maire f. aurea	DBM	Ksabi, near Midelt, C. Morocco	298-1471-68

(*continued on next page*)

Table 1 (*continued*)

Acc.[a]	Taxon (botanical name)[b]	Donor[c]	Collection site[d]	Accession number or name[d]
a61	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	IGB	Bitritto (Bari) near municipal slaugterhouse, Italy	RN20
a62	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	IGB	Bari, road Omodeo-Standa, Italy	RN21
a63	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	IGB	Matera, road Stigliano-Cupolo, Italy	RN24
a64	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	IGB	Matera, road Stigliano-Padula, Italy	RN25
a65	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	IGB	Bitritto (Bari), Italy	RN27
a66	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	IGB	Putignano c/o Lama di Forchia, Italy	RN31
a68	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	IGB	Ege Univ., Faculty of Agriculture-Bornova-Izmir, Turkey	RN35
a69	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	IGB	Bitritto (Bari) in garden near north entrance	RN37
a1	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	SSC	Local selection, Cesena	SA1
a2	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	SSC	Local selection, Cesena	SA2
a3	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	SSC	Local selection, Cesena	SA3
a4	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	SSC	Local selection, Cesena	SA4
a5	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	SSC	Local selection, Cesena	SA5
a6	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	SSC	Local selection, Cesena	SA6
a60	*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Miller) Thell.	DBM	Ruins of Persepolis, Iran	299-3750-75

[a] Accession working number.
[b] According to Gomez-Campo (1999).
[c] DBM: Departamento de Biologia Vegetal, Escuela Tecnica Superior de Ingenieros Agronomos, Universidad Politecnica de Madrid, Madrid, Spain; IGB: Istituto di Genetica Vegetale CNR, Bari, Italy; SSC: Sativa Seeds and Services, Cesena, Italy.
[d] As communicated by donors.

glucosinolate profile, with 51.6% glucoraphenin, together with 16.1% neoglucobrassicin, determining its separation by factor df4.

Clusters c2, c10 and c13 were generated by the splitting of group C. Cluster c2 (*D. harra* a27, a29, a12, and *D. assurgens*) was characterised by the prevalence of sinalbin, and well separated by factor df5. *D. assurgens* showed the presence of glucobrassicin, which is also present in small amounts only in *D. harra* a12.

Cluster c10 (*D. harra* a28) was characterised by positive values of factor df5, for its 46.8% sinalbin, and by factors df9 and df10, respectively negatively and positively correlated to 4-hydroxy glucobrassicin (40% in c10).

D. siifolia a21 represented cluster c13, characterised by over 90% glucobrassicin, strongly negatively correlated to factor df6. Glucobrassicin, and df6, also characterised cluster c5 (*D. tenuifolia* a67 and *D. virgata* a26).

Discriminant factors df1 to df6 explained 99.4% total variance. The other discriminant factors still contributed to cluster differentiation, within group A.

Cluster c1 included almost all the commercial *D. tenuifolia* accessions (a53–a58), many other *E. vesicaria* (a41–a43, a61–a64 and a68–a69) and *D. tenuifolia* (a47–a48, a50–a51), *D. simplex*, *D. cretacea* and *D. muralis* a17. This cluster was characterised by a glucosinolate profile of 7–11 components, with 4-hydroxy glucobrassicin almost always the more abundant, and glucoraphanin at about 20% average content, determining positive values of discriminant factor df7. The almost complete absence of 4-hydroxy glucobrassicin was the main determinant of the clear separation of clusters c6, c9 and c11 from c1, in relation to factor df7.

Cluster c11 (*D. tenuifolia* a34 and *E. vesicaria* a5 and a44) had 2-hydroxyethyl glucosinolate as the main component, a unique trait well defined by the positive values of factor df8. The almost complete absence of glucoraphanin was an additional differential trait with respect to cluster c1.

The commercial *E. vesicaria* accessions (a1–a4, a6), *E. vesicaria* a60 and *D. viminea* represented cluster c6, characterised by positive values of factors df9 and df10, both positively correlated to neoglucobrassicin. Df10 effectively separated cluster c6 from c12, in which this glucosinolate was absent. In *D. viminea*, neoglucobrassicin reached the highest relative content, at 63.2%. *D. viminea* was differentiated from *E. vesicaria* by the lower number of glucosinolates, the absence of glucoraphanin and 4-methoxy glucobrassicin. Glucoerucin was absent in cluster c6, except in *E. vesicaria* a60.

In cluster c9 (*E. vesicaria* a65, a66, a69 and *D. tenuifolia* a49), 4-methoxy glucobrassicin was the relatively more abundant compound, and glucoerucin was present in all accessions. These traits, however, had no clear relation with any discriminant factors. Neoglucobrassicin and 2-hydroxyethyl glucosinolate were present only in *E. vesicaria*.

Cluster c12 (*D. tenuifolia* a35, a46, a52, *D. muralis* a16, and *E. vesicaria* a59) was characterised by the absence of

Table 2
Identified glucosinolate components (g) and HPLC/MS data for desulphoglucosinolates

g.	Chemical name[a]	Common name[a]	Type	Retention time[c]	MS data[c]
g1	2(*S*)-2-Hydroxy-3-butenyl	Epi-progoitrin	Hydroxyalkenyl	4.6	332 *m/z* (M+Na)
g2	4-(Methylsulphinyl)butyl	Glucoraphanin	Alkylthioalkyl	4.7	380 *m/z* (M+Na)
g3	2-Propenyl	Sinigrin	Alkenyl	4.8	302 *m/z* (M+Na)
g4	4-(Methylsulphinyl)-3-butenyl	Glucoraphenin	Alkenylthioalkyl	5.3	378 *m/z* (M+Na)
g5	Ethyl	Glucolepidin	Alkyl	5.6	291 *m/z* (M+Na)
g6	2-Hydroxyethyl	–	Hydroxyalkyl	5.8	306 *m/z* (M+Na)
g7	5-(Methylsulphinyl)-pentyl	Glucoalyssin	Alkylthioalkyl	6.6	394 *m/z* (M+Na)
g8	1-Methylethyl	Glucoputranjivin	Alkyl (branched)	6.8	304 *m/z* (M+Na)
g9	4-Hydroxybenzyl	Sinalbin	Alkylbenzyl	7.0	368 *m/z* (M+Na)
g10	3-Butenyl	Gluconapin	Alkenyl	7.9	316 *m/z* (M+Na)
g11	7-(Methylsulphinyl)heptyl	Glucoibarin	Alkylthioalkyl	8.1	422 *m/z* (M+Na)
g12	4-Hydroxyindol-3-ylmethyl	4-Hydroxyglucobrassicin	Indole	8.7	407 *m/z* (M+Na)
g13	*n*-Butyl	–	Alkyl	9.6	318 *m/z* (M+Na)
g14	Benzyl	Glucotropaeolin	Alkylbenzyl	11.5	352 *m/z* (M+Na)
g15	4-(Methylthio)butyl	Glucoerucin	Alkylthioalkyl	11.8	364 *m/z* (M+Na)
g16	4-Phenylbutyl	–	Alkylbenzyl	12.6	393 *m/z* (M+Na)
g17	Indol-3-ylmethyl	Glucobrassicin	Indole	13.0	391 *m/z* (M+Na)
g18	4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin	Indole	14.0	421 *m/z* (M+Na)
g19	2-Phenylethyl	Gluconasturtiin	Alkylbenzyl	14.7	366 *m/z* (M+Na)
g20	1-Methoxyindol-3-ylmethyl	Neoglucobrassicin	Indole	15.0	421 *m/z* (M+Na)
g21	4-Mercaptobutyl (dimer)[b]	–	Thioalkyl	13.9	675 *m/z* (M+Na)

[a] From Fahey et al. (2001).
[b] From Bennett et al. (2002).
[c] Referred to the analytical conditions reported in the text.

4-hydroxy glucobrassicin; this trait allowed the separation of c12 from the otherwise similar cluster c1, on factor df9.

Hierarchical clustering, based on the presence/absence of glucosinolates (Fig. 3), substantially confirmed the illustrated patterns, with some differences due to the higher impact of quantitatively minor components:

- a clear separation of clusters c1 and c12 from c6, c9 and c11, within visual group A, and a better definition of *D. cretacea*, *D. muralis*, *D. viminea* and *D. simplex* from the bulk *D. tenuifolia* and *E. vesicaria*, within the individual clusters;
- the separation of three *D. harra* (a27, a28 and a29) from the bulk glucosinolate-rich clusters since, although their profile was quantitatively dominated by one component, it also included several minor ones, common to clusters of group A;
- the joining of few accessions from the visual group A (*D. brachycarpa* a23, *D. erucoides* a402, *D. virgata* a26) to some glucosinolate-rich clusters.

3. Discussion and conclusions

3.1. Accession grouping and relations to the phylogeny of the tribe Brassicinae

Previous taxonomic studies of *Diplotaxis* indicated three rather consistent patterns, with reference to haploid chromosome number (*n*):

(a) a group of four $n = 8$ species, endemic of Mediterranean islands and restricted areas of north Africa (*D. siettiana*, *D. brevisiliqua*, *D. ilorcitana* and *D. ibicensis*), rather well defined by molecular (Warwick et al., 1992; Warwick and Black, 1993; Eschmann-Grupe et al., 2003) and biochemical (Sanchez-Yelamo et al., 1992; Sanchez-Yelamo, 1994) markers. In our experience, they represented a well-defined aggregation, within the sinigrin-rich cluster c3.
(b) *D. tenuifolia*, *D. simplex* and *D. cretacea* ($n = 10$), *D. viminea* ($n = 11$) and *D. muralis* ($n = 21$) have been almost invariably identified as a rather homogeneous complex (Sanchez-Yelamo and Martinez-Laborde, 1991; Warwick et al., 1992; Sanchez-Yelamo, 1994; Martin and Sanchez-Yelamo, 2000; Eschmann-Grupe et al., 2003). These taxa represent the bulk of our visual group A, in which more abundant components (glucoraphanin, glucolepidin, glucoerucin, 4-phenyl-butyl glucosinolate) were absent in all the glucosinolate-rich taxa.
(c) *D. brachycarpa* ($n = 9$) was well resolved in most of the literature, except in Eschmann-Grupe et al. (2003). In our experience, this species was rather isolated (cluster c8), the only one with a relevant gluconasturtin content.

The other $n = 10$ (*D. siifolia*), and $n = 9$ (*D. catholica*, *D. assurgens*, *D. virgata* and *D. berthautii*) species were generally not well resolved in previous studies. Warwick et al. (1992) included all of them in one group, subdivided in three sub-groups: (1) *D. assurgens*, *D. tenuisiliqua*, *D. siifolia* and

Table 3
Total glucosinolate content, relative amounts of individual components, group and cluster membership of the accessions examined

Acc.[b]	Species	Glucosinolates[a]																				Group[c]	Cluster[d]
		Total (mg kg^{-1}) dry wt	Relative amount of individual components (g g^{-1})																				
			g1	g2	g3	g4	g5	g6	g7	g8	g9	g10	g11	g12	g13	g14	g15	g16	g17	g18	g19	g20		
a36	*D. cretacea*	1272	–	0.233	–	–	–	–	–	–	0.111	–	–	0.316	–	0.030	0.062	0.170	–	0.078	–	–	A	c1
a17	*D. muralis*	2258	–	0.121	–	–	0.010	–	0.013	0.086	–	–	–	0.294	–	0.023	0.134	0.160	–	0.033	0.014	0.112	A	c1
a30	*D. simplex*	2385	–	0.217	–	–	–	–	0.062	–	–	–	–	0.229	0.031	0.015	0.283	0.120	–	0.030	0.013	–	A	c1
a47	*D. tenuifolia*	3816	–	0.302	–	–	–	–	–	–	–	–	–	0.266	–	0.015	0.280	0.090	0.019	0.019	0.008	–	A	c1
a48	*D. tenuifolia*	2789	–	0.298	–	–	0.012	–	–	–	0.048	–	–	0.191	–	0.023	0.278	0.110	–	0.027	0.013	–	A	c1
a50	*D. tenuifolia*	4647	–	0.233	–	–	–	–	–	–	0.031	–	–	0.327	–	0.015	0.264	0.101	–	0.021	0.010	–	A	c1
a51	*D. tenuifolia*	3158	–	0.267	–	–	0.017	–	–	–	0.065	–	–	0.364	–	–	0.158	0.107	–	–	0.022	–	A	c1
a53	*D. tenuifolia*	2251	–	0.122	–	–	0.019	–	–	–	0.031	–	–	0.493	–	0.026	0.073	0.184	–	0.034	0.017	–	A	c1
a54	*D. tenuifolia*	949	–	0.191	–	–	0.060	–	–	–	0.118	–	–	0.338	–	0.012	0.081	0.201	–	–	–	–	A	c1
a55	*D. tenuifolia*	2582	–	0.168	–	–	0.038	–	–	–	0.073	–	–	0.293	–	0.015	0.181	0.101	–	0.031	0.009	0.091	A	c1
a56	*D. tenuifolia*	2240	–	0.139	–	–	0.020	–	–	–	0.071	–	–	0.312	–	0.016	0.085	0.157	0.093	0.026	0.014	0.067	A	c1
a57	*D. tenuifolia*	2831	–	0.181	–	–	0.025	–	0.043	–	0.069	–	–	0.285	–	0.017	0.133	0.128	–	–	–	0.118	A	c1
a58	*D. tenuifolia*	2420	–	0.182	–	–	–	–	0.046	–	–	–	–	0.295	–	0.015	0.211	0.142	0.059	0.038	0.013	–	A	c1
a41	*E. vesicaria*	2929	–	0.271	–	–	0.017	0.004	–	–	0.033	–	0.006	0.300	–	0.017	0.115	0.153	–	0.024	–	0.061	A	c1
a42	*E. vesicaria*	2081	–	0.215	–	–	0.046	–	–	–	0.061	–	–	0.223	–	–	0.093	0.135	–	–	–	0.226	A	c1
a43	*E. vesicaria*	2682	–	0.156	–	–	0.015	0.013	–	–	0.063	–	–	0.357	–	0.022	0.165	0.141	–	0.026	0.016	0.026	A	c1
a61	*E. vesicaria*	2500	–	0.190	–	–	0.082	0.141	–	–	0.039	–	–	0.203	–	0.017	0.105	0.111	–	–	–	0.112	A	c1
a62	*E. vesicaria*	1325	–	0.127	–	–	0.016	0.007	–	–	0.075	–	–	0.420	–	0.031	0.102	0.222	–	–	–	–	A	c1
a63	*E. vesicaria*	1957	–	0.225	–	–	–	–	–	–	0.052	–	–	0.120	–	0.027	0.173	0.222	–	0.065	0.020	0.096	A	c1
a64	*E. vesicaria*	2413	–	0.174	–	–	–	–	–	–	0.043	–	–	0.294	–	0.018	0.249	0.112	0.022	0.031	0.014	0.045	A	c1
a68	*E. vesicaria*	2186	–	0.175	–	–	0.013	0.009	–	–	0.056	–	–	0.381	–	0.020	0.116	0.117	–	0.051	–	0.061	A	c1
a67	*D. tenuifolia*	3605	–	0.099	–	–	0.010	–	–	–	0.031	–	0.014	0.207	–	0.017	0.026	0.091	0.430	0.025	–	0.049	A	c5
a26	*D. virgata*	2606	–	–	–	–	0.028	–	–	–	0.046	0.200	–	0.110	–	–	–	–	0.522	0.041	0.008	0.044	A	c5
a37	*D. viminea*	816	–	–	–	–	0.147	0.162	–	–	–	–	–	–	–	–	–	0.060	–	–	–	0.632	A	c6
a60	*E. vesicaria*	951	–	0.189	–	–	–	–	–	–	0.121	–	–	–	–	0.026	0.124	0.158	–	0.075	–	0.308	A	c6
a1	*E. vesicaria*	404	–	0.062	–	–	0.152	0.130	–	–	–	–	–	–	–	–	–	0.066	–	0.163	–	0.427	A	c6
a2	*E. vesicaria*	651	–	0.131	–	–	0.117	0.130	–	–	–	–	–	–	–	–	–	0.123	–	0.134	–	0.364	A	c6
a3	*E. vesicaria*	532	–	0.155	–	–	0.115	0.070	–	–	–	–	–	–	–	–	–	0.188	–	0.114	–	0.358	A	c6
a4	*E. vesicaria*	430	–	0.115	–	–	0.123	0.090	–	–	–	–	–	–	–	–	–	0.123	–	0.130	–	0.419	A	c6
a6	*E. vesicaria*	358	–	0.232	–	–	0.111	0.085	–	–	–	–	–	–	–	–	–	0.069	–	0.166	–	0.338	A	c6
a402	*D. erucoides*	3909	–	–	–	0.516	0.010	–	–	–	–	0.022	–	0.119	–	–	0.025	0.114	0.011	0.022	–	0.161	A	c7

a23	*D. brachycarpa*	6091	0.033	–	–	–	0.026	–	–	–	–	0.475	–	–	–	–	–	–	–	–	0.466	–	A	c8
a49	*D. tenuifolia*	248	–	–	–	–	0.149	–	–	–	–	–	–	–	–	–	0.147	0.342	–	0.362	–	–	A	c9
a65	*E. vesicaria*	1801	–	0.102	–	–	–	0.022	–	–	0.033	–	–	0.147	–	0.015	0.075	–	–	0.570	–	0.036	A	c9
a66	*E. vesicaria*	322	–	0.115	–	–	0.127	0.087	–	–	–	–	–	–	–	–	0.046	0.137	–	0.316	–	0.172	A	c9
a69	*E. vesicaria*	351	–	–	–	–	0.156	0.090	–	–	–	–	–	–	–	0.142	0.108	–	–	0.303	–	0.200	A	c9
a34	*D. tenuifolia*	640	–	0.066	–	–	0.230	0.324	–	–	–	–	–	–	–	–	0.121	0.124	–	–	–	0.135	A	c11
a44	*E. vesicaria*	1762	–	–	–	–	0.141	0.416	–	–	–	–	–	–	–	–	–	0.012	–	0.028	–	0.403	A	c11
a5	*E. vesicaria*	691	–	–	–	–	0.076	0.634	–	–	–	–	–	–	–	–	–	–	–	–	–	0.290	A	c11
a16	*D. muralis*	1027	–	0.235	–	–	0.031	–	–	0.138	–	–	–	–	–	0.033	0.171	0.279	–	–	0.009	0.104	A	c12
a35	*D. tenuifolia*	1151	–	0.350	–	–	–	–	–	–	0.090	–	–	–	–	–	0.304	0.256	–	–	–	–	A	c12
a46	*D. tenuifolia*	1384	–	0.346	–	–	0.042	–	–	–	0.062	–	–	–	0.183	0.058	0.309	–	–	–	–	–	A	c12
a52	*D. tenuifolia*	835	–	0.397	–	–	0.060	–	–	–	0.194	–	–	–	–	–	–	0.318	–	–	0.031	–	A	c12
a59	*E. vesicaria*	1254	–	0.197	–	–	0.022	–	–	–	0.055	–	–	–	–	0.024	0.274	0.230	–	0.075	0.020	0.102	A	c12
a15	*D. berthautii*	6563	–	–	0.947	–	0.003	–	–	–	–	–	–	–	0.019	–	–	–	–	0.014	–	0.017	B	c3
a39	*D. brevisiliqua*	10257	–	–	1.000	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	B	c3
a401	*D. erucoides*	8358	–	–	1.000	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	B	c3
a13	*D. ibicensis*	6409	–	–	0.835	–	0.006	0.001	–	–	–	–	–	0.076	–	–	–	–	0.035	0.018	–	0.028	B	c3
a18	*D. ilorcitana*	15912	–	–	0.953	–	0.003	–	–	–	–	–	–	0.016	–	–	–	–	0.012	0.011	–	0.005	B	c3
a381	*D. ollivieri*	12946	–	–	0.932	–	0.004	–	–	–	0.004	–	–	–	–	–	–	–	0.017	0.011	–	0.033	B	c3
a19	*D. siettiana*	9433	–	–	0.896	–	0.003	–	–	–	–	–	–	0.062	–	–	–	–	0.008	0.009	–	0.021	B	c3
a24	*D. tenusiliqua*	15262	–	–	0.991	–	0.005	0.004	–	–	–	–	–	–	–	–	–	–	–	–	–	–	B	c3
a32	*D. virgata*	10799	–	–	1.000	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	B	c3
a25	*D. catholica*	6891	0.010	–	–	–	0.037	–	–	–	0.006	0.903	–	–	–	–	–	–	0.036	0.001	0.007	–	B	c4
a382	*D. ollivieri*	13740	0.004	–	0.003	–	0.011	–	–	–	–	0.809	–	–	–	–	0.007	–	0.121	0.012	–	0.033	B	c4
a20	*D. siifolia*	11228	–	–	–	–	0.022	0.005	–	–	0.014	0.798	–	0.019	0.006	–	–	–	0.135	–	–	–	B	c4
a22	*D. siifolia*	6702	–	–	–	–	0.034	–	–	–	–	0.923	–	–	–	–	–	–	0.012	0.017	–	0.014	B	c4
a31	*D. virgata*	9996	0.008	–	–	–	–	–	–	–	–	0.831	–	0.022	–	–	0.035	–	0.093	0.011	–	–	B	c4
a12	*D. harra*	15948	–	–	–	–	–	–	–	–	0.823	0.027	–	0.051	–	–	–	–	0.031	0.054	–	0.013	C	c2
a27	*D. harra*	31554	–	–	0.002	–	0.002	–	–	–	0.846	0.006	–	0.023	–	–	–	0.001	–	0.121	–	–	C	c2
a29	*D. harra*	32357	–	–	0.020	–	–	–	–	–	0.853	–	–	0.014	–	–	–	0.001	–	0.112	–	–	C	c2
a141	*D.assurgens*	17728	0.120	–	–	–	–	–	–	–	0.601	0.012	–	0.050	–	–	0.002	0.001	0.128	0.077	–	0.009	C	c2
a142	*D.assurgens*	23021	0.049	–	–	–	0.002	–	–	–	0.706	0.002	–	0.032	–	–	–	–	0.146	0.058	–	0.005	C	c2
a28	*D. harra*	14903	–	0.008	–	–	0.003	0.002	0.006	–	0.468	0.042	–	0.396	–	–	–	0.007	–	0.069	–	–	C	c10
a21	*D. siifolia*	74063	0.036	–	–	–	–	–	0.004	0.005	–	0.004	–	0.006	0.003	–	–	–	0.915	0.002	–	0.023	C	c13
	LSD (p = 0.05)	*4208*	*0.006*	*0.053*	*0.004*	*0.021*	*0.038*	*0.036*	*0.006*	*0.001*	*0.039*	*0.008*	*0.002*	*0.068*	*0.016*	*0.026*	*0.034*	*0.025*	*0.026*	*0.020*	*0.005*	*0.063*	–	–

[a] See Table 2 for compound identification.
[b] Accession working number.
[c] Groups visually individuated in Fig. 1.
[d] On the basis of non-hierarchical clustering and discriminant analysis.

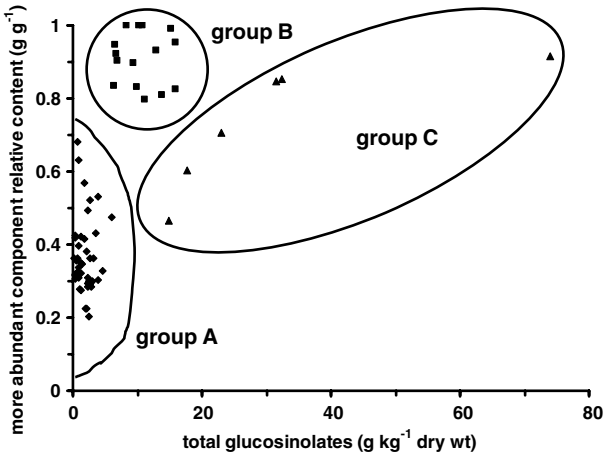

Fig. 1. Relations between total glucosinolate content and the relative amount of the more abundant component. The three groups are visual aggregations of accessions.

one accession of *D. virgata*; (2) *D. berthautii* and *D. virgata* f. *sahariensis*; (3) *D. catholica* and *D. virgata* subsp. *virgata*. Almost the same aggregations were reported from later studies (Warwick and Black, 1993; Warwick and Sauder, 2005). *D. siifolia*, *D. assurgens*, *D. catholica* and *D. tenuisiliqua* were grouped together on the basis of reserve seed proteins (Sanchez-Yelamo and Martinez-Laborde, 1991), including also *D. harra*. *D. assurgens*, *D. virgata* and *D. tenuisiliqua* were rather well grouped by their flavonoid profiles, but the similarity between them was rather low (Sanchez-Yelamo, 1994). Inter simple sequence repeat markers (ISSR) linked *D. catholica* and *D. virgata* at a high similarity level, in a group also including *D. siettiana* and *D. harra* (Martin and Sanchez-Yelamo, 2000). Finally, Eschmann-Grupe et al. (2003) found a high degree of heterogeneity in the Random Amplified Polymorphic DNA (RAPDs) profiles of these species.

Our results confirm the similarity between some of the $n = 9$ and $n = 10$ species, but clearly separated others on the basis of glucosinolate profile. In fact, *D. berthautii*, *D. tenuisiliqua, D. virgata* a32, and *D. ollivieri* a381were included in the sinigrin-rich cluster c3. *D. siifolia*, *D. catholica*, *D. virgata* a31 and *D. ollivieri* a382 belonged to the gluconapin-rich cluster c4. Traditional taxonomy classified these taxa in the section *Rhynchocarpum* (Gomez-Campo, 1999), including also *D. assurgens. D. harra*, on the contrary, was the only representative of subgenus *Hesperidium* considered in this research.

Molecular methods indicated two probable early-diverging evolutionary lines, within the tribe Brassicinae, named respectively *rapaloleracea* and *nigra* lineages (Warwick and Black, 1991, 1993; Pradhan et al., 1992; Warwick et al., 1992). Major genera, including *Diplotaxis*, were represented in both lineages, and therefore considered as somewhat artificial groups, of likely polyphyletic origin. This scheme, although not fully supported by some studies (Warwick and Sauder, 2005), was maintained in more recent taxonomic syntheses of the *Brassica* coenospecies complex (Gomez-Campo and Prakash, 1999), or the whole family (Al-Shehbaz et al., 2006). No research trying to connect biochemical traits with this classification has been carried out previously.

Our results indicate that most taxa included in the *nigra* lineage are glucosinolate-rich species, strongly characterised by one dominant component. These were rather clearly separated by both clustering procedures, and included: (a) all the accessions of the visual group B, and subsequent clusters c3 and c4; (b) some accessions of group C: *D. assurgens*, and *D. siifolia* a21; (c) *D. brachycarpa* (group A, cluster c8). The positions of *D. erucoides* and *D. harra*, included in the *rapaloleracea* lineage in most previous studies, will be further discussed; in some researches, however, these species were found closer to cytodemes of the *nigra* lineage (Martin and Sanchez-Yelamo, 2000).

3.2. Specific and sub-specific taxonomy in relation to glucosinolates

The taxonomy of some *Diplotaxis* species has been revised several times, but remains problematic even after molecular marker investigations.

A yellow flowered *D. erucoides* type, endemic to North Africa, was classified as a subspecies (*D. erucoides* (L.) DC subsp. *cossoniana* (Reut. ex. Boiss.) Martinez-Laborde) by Martinez-Laborde (1991). Warwick et al. (1992) also characterised it and, in a later paper (Warwick and Sauder, 2005), re-introduced the specific rank of *D. cossoniana* (Reut. ex Boiss) O.E. Schulz. The white and yellow flowered types also differed for flavonoid profiles, and the yellow flowered form was reported as *D. erucoides* (L.) DC subsp. *longisiliqua* (Cosson) Gomez-Campo (Sanchez-Yelamo, 1994). In our study the two forms were rather distant: in fact, the white flowered individuals of the accession a401 belonged to the sinigrin-rich cluster c3, and the yellow flowered plants (a402) to the rather isolated cluster c7, having glucoraphenin as an exclusive component.

D. virgata has been recognised as one of the more problematic species, as previously illustrated, with several subspecies described, and retained in the recent taxonomic reviews (Gomez-Campo, 1999). Glucosinolate profiles confirmed this situation: in fact, *D. virgata* subsp. *rivulorum* (a26) was a low-glucosinolate taxon, although with a peculiar profile, characterised by glucobrassicin and gluconapin; *D. virgata* subsp. *australis* (a32) belonged to the sinigrin cluster c3, whereas *D. virgata* subsp. *virgata* (a31) belonged to the gluconapin-rich cluster c4, together with *D. catholica*.

D. siifolia subsp. *vicentina* (a21) was rather different from the other two accessions, because of its very high glucosinolate content, the dominance of glucobrassicin, and a different leaf morphology. It has sometimes been considered as an independent species (*D. vicentina* (Sampaio) Rothm.), although Martinez-Laborde (1992) suggested maintaining its sub-specific status. Our results support its elevation at the specific rank. On the other hand, *D. harra* subsp. *confusa* (a28), although placed in a

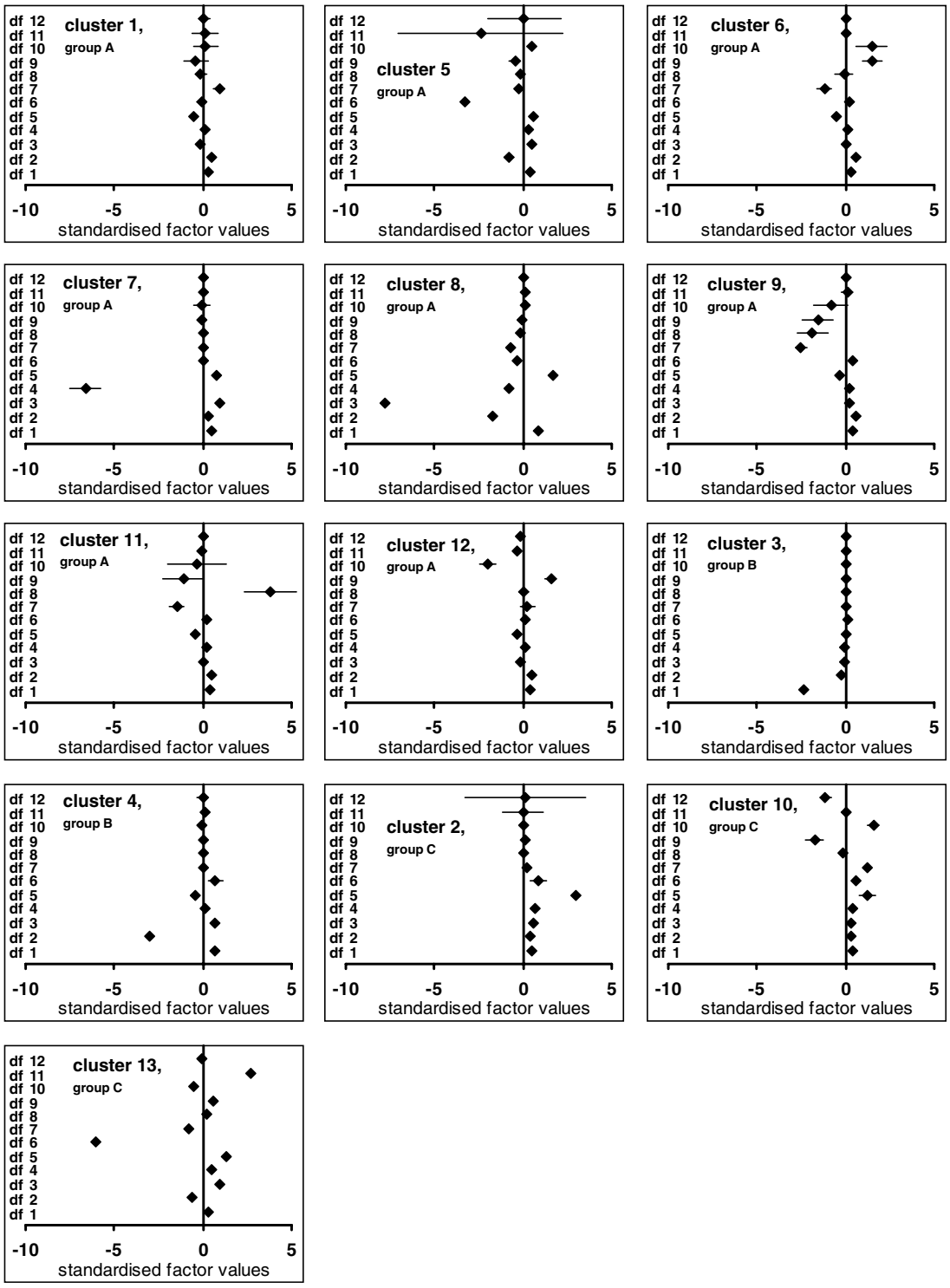

Fig. 2. Standardised discriminant factor (df) profiles of the 13 clusters individuated by means of non-hierarchical clustering. Horizontal bars: within-cluster factor standard deviation.

different cluster, shared the main component (gluconapin) with the other three accessions of the same species.

The genus *Eruca* is considered as mono-specific, with three subspecies. In our experience, the separation of *E. vesicaria* accessions in the low-glucosinolate clusters c1, c6, c9, c11 and c12 does not seem to have any relation with the sub-specific classification.

3.3. Relations to known biosynthetic pathways

Glucosinolate biosynthetic pathways have been periodically revised (Halkier and Du, 1997; Mithen et al., 2000; Mithen, 2001; Fahey et al., 2001; Wittstock and Halkier, 2002; Grubb and Abel, 2006; Halkier and Gershenzon, 2006). Although none of the contributions dealt directly

Table 4
Correlations among glucosinolate content and linear discriminant factors derived from backward selection procedure (13 compounds retained), for the 13 clusters individuated by means of non-hierarchical clustering

Compound	Correlation to discriminant factors[a]											
	df1	df2	df3	df4	df5	df6	df7	df8	df9	df10	df11	df12
g1	0.09	−0.04	0.11	0.12	0.51	−0.42	−0.04	0.02	0.08	−0.06	0.40	0.50
g2	0.27	0.34	−0.10	0.09	−0.39	−0.09	0.52	−0.11	0.00	−0.15	−0.13	0.09
g3	−0.94	−0.10	−0.03	−0.01	0.03	0.04	−0.01	0.01	0.01	−0.01	−0.01	−0.01
g4	0.07	0.04	0.14	−0.95	0.11	0.00	0.00	0.01	−0.01	−0.03	0.00	0.00
g6	0.09	0.13	0.01	0.05	−0.13	0.07	−0.33	0.74	−0.20	0.05	0.01	0.00
g9	0.13	0.11	0.17	0.18	0.84	0.26	0.08	0.00	0.00	0.02	−0.08	−0.20
g10	0.23	−0.89	0.09	0.02	−0.08	0.16	0.00	0.00	0.02	0.00	0.06	−0.02
g11	0.05	−0.06	0.05	0.03	0.04	−0.37	0.01	−0.03	−0.09	0.08	−0.86	0.28
g12	0.15	0.16	0.06	0.04	0.20	−0.02	0.43	−0.08	−0.36	0.25	0.00	−0.04
g17	0.04	−0.10	0.13	0.06	0.20	−0.72	−0.09	0.02	0.07	−0.06	0.32	0.02
g18	0.12	0.10	0.17	0.16	0.75	0.24	−0.01	−0.10	−0.08	0.03	−0.07	−0.22
g19	0.11	−0.20	−0.94	−0.10	0.18	−0.04	−0.07	−0.02	−0.01	0.01	0.02	0.00
g20	0.05	−0.03	0.19	−0.23	0.10	−0.55	−0.19	0.18	0.19	0.22	0.23	0.04
% EV [b]	*39.9*	*26.5*	*12.7*	*11.3*	*6.3*	*2.7*	*1.4*	*0.6*	*0.3*	*0.2*	*0.02*	*0.002*

[a] Critical significant levels: $P = 0.05{:}0.16$; $P = 0.01{:}0.21$; $P = 0.001{:}0.27$.
[b] % EV: percentage of variance explained by each factor.

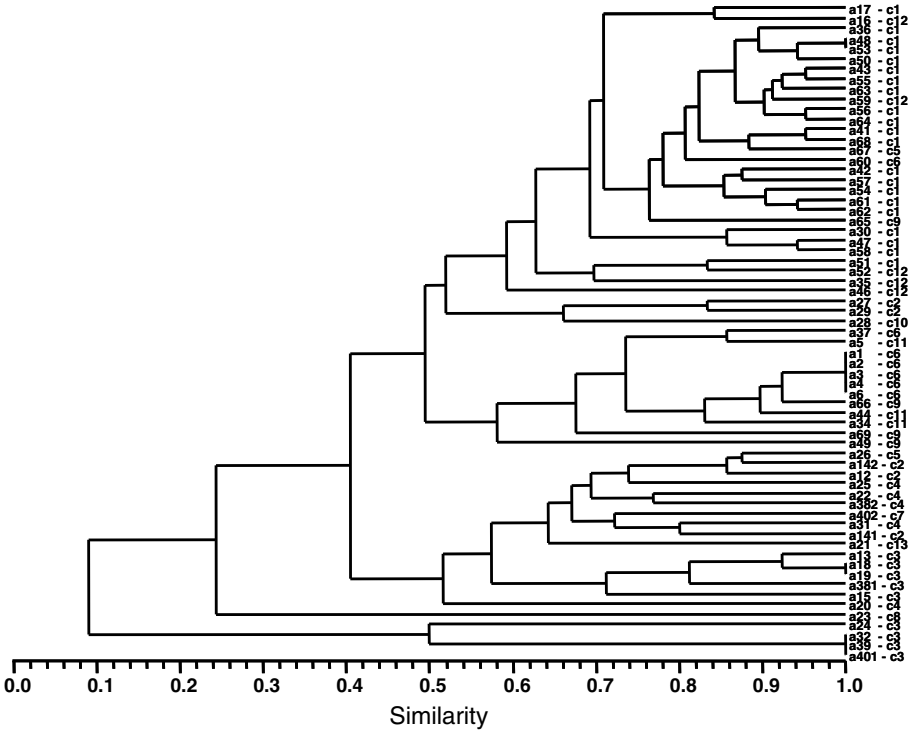

Fig. 3. Dendrogram representation of the UPGMA cluster analysis of Dice similarity coefficients, calculated on the presence/absence data of glucosinolate components. Dendrogram lines are labelled with accession (a) and non-hierarchical cluster (c) identifiers.

with taxonomy, some links can be made to the interpretation of our results on *Diplotaxis* and *Eruca*.

Sinigrin and gluconapin, characterising the bulk of glucosinolate-rich accessions (clusters c3 and c4), in the *nigra* lineage, are clearly related. They differ in only one step in the methionine chain elongation, but have the same side chain structure, with a methylene group in the terminal position. Although the specificity of methionine elongation biosynthetic pathways is still being investigated (reviews in Grubb and Abel, 2006, and Halkier and Gershenzon, 2006), the hypothesis of genetic affinity of most accessions belonging to the *nigra* lineage is supported by their glucosinolate profiles.

D. muralis, an allotetraploid of likely derivation from *D. tenuifolia* and *D. viminea* (Sanchez-Yelamo et al., 1992; Martin and Sanchez-Yelamo, 2000) had the unique presence of glucoputranjivin, which is absent from both its putative parents. Glucoputranjivin in *D. viminea* was

reported by Fahey et al. (2001). Glucoputranjivin is a branched-chain compound, of possible direct derivation from valine, one of the glucosinolate precursor amminoacids (Mithen, 2001); in fact, no secondary biosynthetic pathways involving the branching of methionine-derived glucosinolates are reported. The use of valine as a precursor seems therefore to be exclusive of *D. muralis*, among the material observed. The synthesis of compounds not present in parental lines has been detected also in *Arabidopsis thaliana* (Kliebenstein et al., 2001a).

Sulphur-containing side chain, methionine-derived components (glucoraphanin, glucoalyssin, glucoibarin and glucoerucin) were exclusive to group A accessions, representing the bulk of the *rapaloleracea* lineage species. This group also had relevant percentages of tryiptophan-derived indolic glucosinolates, the specificity of which, interestingly, was the main determinant of within-group cluster discrimination. The additional presence of phenylalanine-derived aromatic compounds indicates the capability of exploiting different pathways in the initial steps of glucosinolate biosynthesis. Also this complexity is similar to that reported for *Arabidopsis* (Halkier and Du, 1997; Kliebenstein et al., 2001a,b; Grubb and Abel, 2006). On the other hand, modified side chain methionine-derived components were less frequent in group A.

Accessions of clusters c2 and c10 are rather specialised in the biosynthesis of phenylalanine-derived sinalbin, although this component is not exclusive to these taxa.

3.4. General conclusions and applied aspects

Mature leaves have been used for the first time in this extensive germplasm exploration, in contrast to the use of seeds in previous investigations (Daxenbichler et al., 1991; Tsukamoto et al., 1993).

From a taxonomic point of view, this research contributes to the knowledge of glucosinolate expression not previously investigated in the genus *Diplotaxis*. Substantial congruence with the current systematics was observed, providing additional information on critical taxa. In particular, all the species with a glucosinolate profile well characterised by the prevalence of one component were clearly discriminated from the others. Among them, *D. harra* remained rather isolated, confirming its position in a separate subgenus.

The *D. tenuifolia* and *E. vesicaria* accessions were clustered on the basis of different combinations of indolic components, and the presence and relative abundance of glucoraphanin.

From an applied aspect, this research contributes to the evaluation of germplasm accessions held in gene banks, for potentially useful characters.

In this respect, types with extremely high glucosinolate content in the vegetative parts, and a profile represented mostly by one component, mainly discovered within *D. siifolia* and *D. harra*, may be of potential interest for either the health sector or biofumigation.

Ample variability of glucosinolate profiles of edible *D. tenuifolia* and *E. vesicaria* was detected, corresponding to a prevalence of methionine derived components, but also including benzylic and indolic glucosinolates. We envisage the possibility of breeding types with improved composition and taste in these taxa, and in *D. muralis*.

4. Experimental

4.1. Plant material

Sixty-three accessions of *Diplotaxis* and *Eruca* were considered (Table 1). Voucher specimens are held by the institutions that supplied the seeds. Digital images, taken at the main developmental stages, are held in our electronic databases.

The seeds were planted in alveolated plates, with peat/sand substrate, in a unheated glasshouse, on March 15, 2004; the plantlets were transplanted in the open field at the fourth-fifth true leaf stage, at the Martorano 5 experimental farm, near Cesena, Emilia Romagna, northern Italy, on April 29, 2004. Each accession was planted in an unreplicated plot of four rows, 20 cm apart.

Mature leaves were harvested at beginning of flowering, between June 6 and 12, from 10 plants from the central two rows of the plot. Leaf samples were frozen immediately and then freeze dried. A sub-sample was used for the determination of dry matter.

4.2. Glucosinolate extraction and analysis

Desulphoglucosinolates were analysed according to Schuetze et al. (1999), with slight modifications. 200 mg freeze-dried samples (three replications per accession) were heated for 4 min at 75 °C in a dry heat (Thermoblock Falc 120), in order to inactivate the endogenous myrosinase. The extraction was carried out with the addition of 3 ml 70% plus 3 ml 10% methanol (after 4 min), at 75 °C for 15 min with stirring. After centrifugation at 4500 rpm for 5 min, 750 μl of the extract were injected into a SPE column containing a DEAE Sephadex anion exchanger (A-25, Sigma), preliminarily washed with 2 ml Na-acetate buffer (pH 4.0). After the addition of 120 μl diluted sulphatase (Sigma S9626), the samples were incubated at 39 °C for 16 h, to allow the enzymatic desulphatation of glucosinolates. The desulpho-glucosinolates were washed three times with 350 μl HPLC grade water, filtered through a 0.45 μm filter, and stored at −18 °C until HPLC analysis. HPLC separation was carried out on a Zorbax Eclipse XDBC18 column (3.0 × 150 mm, 3.5 μm) under the following conditions: injected volume 20 μl, flow rate 0.550 ml/min, column temperature 30 °C, wavelength detection 229 nm. Elution was carried out with a water

(A) – acetonitrile (B) gradient, as follows: start with 99% A, linear gradient to 75% A at min 17.5, linear gradient to 99% A at min 20; total analysis time: 35 min. The individual glucosinolates were identified by means of a HPLC-MS 1100 Series (Agilent), with the same analytical conditions described. The instrument was configured for atmospheric pressure electrospray ionisation (API-ESI), with positive polarity, acquisition mode scan. Full scan spectra were acquired over the range 50–800 *m/z*. The content of glucosinolates was quantified using sinigrin (Sinigrin hydrate, 85440 Fluka) as an external standard, and considering the relative response factors of the individual glucosinolates (Wathelet et al., 1991).

4.3. Statistical analysis

The main part of statistical analysis was carried out by means of non-hierarchical clustering (*K*-means method) applied at the relative abundance of individual glucosinolate components. This approach was chosen since no real hierarchical structure was present in the original data. Confirmatory linear discriminant analysis was applied at each step, until no further reduction of between-group variability, or increment of the discriminant power of individual components, was achieved for further cluster division. A backward selection procedure of variables was adopted in final discriminant analysis, to retain only the most relevant glucosinolate components for cluster membership discussion. Individual replications were retained during these analyses. All analyses were carried out by means of the SYSTAT® package.

An additional clustering procedure was carried out, based on the presence/absence of individual glucosinolates. In this case, the UPGMA clustering algorithm was applied at the Dice coefficients similarity matrix, by means of the NTSYSpc® statistical package. This approach is more similar to that commonly adopted in biochemical or molecular marker taxonomy, and may have the advantage of reducing the weight of high relative abundance values on cluster individuation.

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