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# Genetic relationships among species of the genus *Diplotaxis* (*Brassicaceae*) using inter-simple sequence repeat markers

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**Abstract** Inter-simple sequence repeat (ISSR) amplification was evaluated for its applicability as a genetic marker system to establish relationships among ten *Diplotaxis* species. ISSR amplification generated multiple banding profiles with the 12 primers from all DNA samples, with an average of 41.2 fragments per primer. This average was clearly higher for the 5´ triple-anchored primers than for other primers. The banding profiles were highly repeatable across separate PCR runs. DNA mixing procedures were found to be appropriate strategies to generate banding patterns representative of each species studied. Similarity values were calculated considering 494 ISSR bands, and a dendrogram was constructed based on the similarity matrix. The ten *Diplotaxis* species were clustered into two major groups. The first group consists of five species, *Diplotaxis tenuifolia* and *Diplotaxis cretacea*, and *Diplotaxis muralis* with their putative parents (*D. tenuifolia* and *Diplotaxis viminea*). In the second group three species are clustered that are closely related (*Diplotaxis virgata*, *Diplotaxis catholica* and *Diplotaxis siettiana*), in addition to *Diplotaxis harra*, and *Diplotaxis erucoides*, which has lowest similarity values with the rest of the species studied. The two groups defined in the present work may be concordant with the idea suggested by several authors of a biphyletic origin for *Diplotaxis*. The genetic relationships among the ten *Diplotaxis* species estimated by the polymorphism of ISSR markers are in agreement with those previously inferred by other morphological, biochemical and molecular data, indicating the reliability of the ISSR approach for this purpose.

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## Introduction

The genus *Diplotaxis* DC. is native to the Mediterranean region and comprises about 30 species (Gómez-Campo 1999), distributed in Central Europe and the Mediterranean area, particularly from the Iberian peninsula and northwestern Africa (with several endemic taxa). Some of them extend eastward to India and Pakistan, five other species are endemic to the Cape Verde Islands and another one was described from western Nepal. Also, representative taxa of the genus are naturalised in the New World (Al-Shehbaz 1985). *Diplotaxis* belongs to the tribe *Brassiceae*, subtribe *Brassicinae* (family: *Brassicaceae*), and is considered to be a genus which exhibits nearly all the primitive morphological characters of the tribe (Gómez-Campo 1980). Although the genus has no economic importance, however it is one of the nearest wild relatives of the economically important genus *Brassica*. This close relationship was reflected from a cytogenetical point of view by Harberd (1972), and included several species of *Diplotaxis* in the *Brassica* "coenospecies", defined as wild taxa sufficiently related to the cultivated species of *Brassica* and capable of experimental hybridization with them. Therefore, *Diplotaxis* constitutes a potential genetic resource for use in plant breeding programs (Gómez-Campo 1980, 1999), and there are several reports where wild relatives are used as a valuable source of genes for crop improvement (Rao et al. 1994; Malik et al. 1999). Within the genus, the gametic chromosome numbers are 7, 8, 9, 10, 11 and 13. Natural interspecific hybrids in *Diplotaxis* are rare; however, based on morphological and cytological data, the allopolyploid origin of *Diplotaxis muralis* (L.) DC (n=21), derived from *Diplotaxis tenuifolia* (L.) DC (n=11) and *Diplotaxis viminea* (L.) DC (n=10), was presented by Harberd and McArthur (1972).

Studies of the characterisation of, as well as the establishment of taxonomic relationships between, species of

the genus *Diplotaxis* have been realised using different markers and methodologies including: morphological traits (Martínez-Laborde 1991a, b; Gómez-Campo and Martínez-Laborde 1998); biochemical markers as flavonoids (Sánchez-Yélamo and Martínez-Laborde 1991; Sánchez-Yélamo 1994), seed proteins (Sánchez-Yélamo et al. 1992) and seed isozymes (Sánchez-Yélamo and Martínez-Laborde 1991); and molecular markers using chloroplast and mitochondrial DNA (by the RFLP technique; Pradhan et al. 1992; Warwick et al. 1992). However, the genetic relationships within the genus *Diplotaxis* have never been studied using nuclear DNA markers.

Tandemly repetitive DNA sequences, such as mini- and micro-satellites, are a major component of all eukaryotic genomes. Wang et al. (1994) found that dinucleotide microsatellites are prevalent in plants while mono- tri- and tetra-nucleotide repeats are less common. Microsatellites or simple sequence repeat (SSR) can be detected by PCR (sequence-tagged microsatellite-site methodology) and they provide co-dominant markers with a high degree of allelic polymorphism. However, these markers can only be obtained through a difficult and labour-intensive procedure, principally because they require prior DNA sequence information for primer design. Recently, a relatively novel molecular technique that permits the detection of polymorphisms in microsatellite and inter-microsatellite loci without previous knowledge of the DNA sequence has been described: 'inter-simple sequence repeat PCR' (ISSR-PCR, or ISSR) (Zietkiewicz et al. 1994), also named 'random amplified microsatellite polymorphisms' (RAMPs) by Wu et al. (1994). This technique involves the use of a single primer composed of a microsatellite sequence plus a short arbitrary sequence (anchor), which targets a subset of SSRs or microsatellites and amplifies the region between two closely spaced and oppositely oriented simple sequence repeats. The production of large numbers of fragments, reproducibility, and low cost are advantages of these ISSR markers (Salimath et al. 1995; Fang et al. 1997; Moreno et al. 1998).

Wolfe and Liston (1998) reviewed the literature on ISSR applications and found that the majority of ISSR markers studies have been restricted to cultivated species and at the intraspecific level. Consequently, this technique has been used widely for DNA fingerprinting and assessing genetic diversity in closely related germplasm, principally of cultivated plants such as oilseed rape (Charters et al. 1996), trifoliate orange (Fang et al. 1997), grapevine (Moreno et al. 1998), lupin (Gilbert et al. 1999), and rice (Blair et al. 1999). Likewise, the use of these markers in natural plant populations have shown their great potential for population-level and interspecies-level studies (Wolfe and Liston 1998; Wolfe et al. 1998). This applicability of ISSR-PCR for genomic fingerprinting at the interspecies-level and for inferring genetic relationships among related species has previously been indicated by Zietkiewicz et al. (1994). However, the ISSR markers have been tested at subspecies or species levels in only a few known examples namely: to distinguish between two subspecies of *Plantago major* (Wolff and Morgan-Richards 1998); to study hybridization in natural populations from the genera *Fallopia* (Hollingsworth et al. 1998) and *Penstemon* (Wolfe et al. 1998); as a genetic marker system in diploid, tetraploid and hexaploid wheat species (Nagaoka and Ogihara 1997); and to establish genetic relationships among species in the genera *Eleusine* (Salimath et al. 1995) and *Citrus* (Fang et al. 1998).

DNA mixing procedures can be effective strategies for assessing large quantities of plant material or for detecting genetic markers characterising different groups or taxa (Charters et al. 1996; Furman et al. 1997; Gilbert et al. 1999). This method of sampling provides a better representation of a group or taxon than using DNA from a single plant only (Virk et al. 1995).

The present study is the first report of the applicability of the nuclear DNA marker ISSR-PCR in characterising genetic affinities at the interspecies level in the genus *Diplotaxis*. Our results permit us to establish genetic relationships among ten related species within the genus *Diplotaxis*, and show that the ISSR approach is a interesting tool for plant genome analysis.

## Materials and methods

### Plant material

Ten species of the genus *Diplotaxis* were analysed in this study (Table 1). Five individuals per taxon were used. The *Diplotaxis* species were not compared with an outgroup species since the purpose of this investigation was to establish interspecific relation-

**Table 1** *Diplotaxis* species studied, gametic number and origin of samples

| Taxon | Gametic number | Accession number[a] | Origin[b] |
|---|---|---|---|
| *D. tenuifolia* (L.) DC. | 11 | (–) | Madrid, Spain |
| *D. muralis* (L.) DC. | 21 | 990 | BG Berlin, Germany |
| *D. viminea* (L.) DC. | 10 | 2108 | BG Munich, Germany |
| *D. cretacea* Kotov | 11 | 4189 | BG Moscow, Russia |
| *D. simplex* (Viv.) Sprengel | 11 | 1931 | Ain Sefra, Algeria |
| *D. harra* (Forsk.) Boiss. | 13 | 1939 | Ain Sefra, Algeria |
| *D. virgata* (Cav.) DC. | 9 | (–) | Madrid, Spain |
| *D. catholica* (L.) DC. | 9 | (–) | Madrid, Spain |
| *D. erucoides* (L.) DC. | 7 | (–) | Madrid, Spain |
| *D. siettiana* Maire | 8 | 3025 | Alboran Island, Spain |

[a] At the germplasm bank of the 'Departamento Biología Vegetal, E.T.S.I. Agrónomos de Madrid, Spain'. (–) indicates plant material collected in the natural habitats

[b] BG=Botanic Garden

ships and not strictly phylogenetic relationships. Fresh leaves were collected from plants in the field or from seedlings grown from seeds stored under long-term conservation conditions at the germplasm bank of the 'Departamento Biología Vegetal, E.T.S.I. Agrónomos de Madrid, Spain'. These leaves were frozen and transferred to the freezer at –80°C.

### DNA extraction, amplification and electrophoresis

Genomic DNA was extracted from frozen young leaves of single individuals following Torres et al. (1993). Extracted DNA was quantified using a spectrophotometer, and comparing band intensities with know standards of lambda DNA on 1% agarose gels. A working solution of DNA (10 ng/µl) was made with sterile double-distilled water.

In a preliminary study, a total of 22 ISSR primers were chosen from the 100 provided in primer set #9 of the University of British Columbia Biotechnology Laboratory (UBC, Vancouver, Canada), and were tested for a subset of four species (*D. tenuifolia, D. muralis, D. viminea* and *Diplotaxis erucoides*). These primers were selected by their different structure (core repeats and anchored nucleotides): primers based on dinucleotide repeats anchored by one nucleotide or by two partially degenerate nucleotides at the 3´ end, or by three degenerate nucleotides at the 5´ end; and tri-, tetra- and penta-nucleotides repeats without an anchor. Finally, we selected 12 primers (see Table 2), by their number and consistency of amplified fragments, for analysing all individuals of the ten species.

DNA amplifications were performed in a 20-µl reaction volume containing about 30 ng of template DNA, 0.5 µM of a single primer (UBC, Vancouver, Canada), 200 µM of each dNTP and 0.5 units of BIOTAQ DNA polymerase in the buffer (10× with 25 mM $MgCl_2$) provided by the manufacturers of the enzyme (Bioprobe systems). The reaction mix was overlaid with a drop of mineral oil. Amplifications were performed in a PTC-100 thermal cycler (MJ Research, Inc.) programmed for an initial step of 1 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 52°C and 2 min at 72°C, and a final 5-min extension at 72°C.

From the 20 µl obtained after each amplification, aliquots of 8.5 µl were separated in 2% agarose (NuSieve 3:1, FMC BioProducts) gels, in 1X TBE buffer, at 3 V/cm for 4 h, and stained with ethidium bromide (0.5 µg/ml). The gels were visualized and photographed under UV light. The molecular size of fragments was estimated by reference to a 100-bp ladder (Pharmacia). At least two PCR amplifications were done for each sample, and only reproducible bands in several runs were considered for analysis.

For comparison we have used two DNA mixing strategies to detect genetic patterns representative from each of the ten species studied. In the template-mixing strategy, equal amounts of working solution DNAs from each five individuals of the same species were pooled as the 'species template DNA' prior to the PCR reaction. The product-mixing strategy consisted of mixing equal volumes of separately generated PCR products from each five individuals of the same species prior to electrophoresis.

### Data analysis

The banding patterns obtained with DNA mixing strategies were analysed to estimate genetic relationships among the ten *Diplotaxis* species using ISSR markers. In these species-patterns only the fragments amplified at least in three of the five individuals used for each species were considered. The ISSR bands were interpreted as dominant markers and were scored as diallelic characters either as 1 (present) or 0 (absent). A pair-wise similarity matrix was calculated using the simple matching coefficient. This similarity matrix was employed to construct a dendrogram by the unweighted pair group method with arithmetical averages (UPGMA), using the SAHN-clustering and TREE programs from the NTSYS-pc, version 1.6 package (Rohlf 1992). A co-phenetic matrix was derived from the similarity matrix to test the goodness of fit of the clusters by comparing the two matrices using the Mantel matrix correspondence test in the MXCOMP program of the NTSYS-pc package.

## Results and discussion

### ISSR analysis

ISSR amplifications using 12 primers generated a total of 494 reliable fragments from the product-mixing patterns of the ten *Diplotaxis* species. The size of these fragments ranged between 230 and 1500 bp (Table 2). Among the ten species of *Diplotaxis* analysed all 494 bands were polymorphic, and only one band was simultaneously present in nine species. Similarly, 96% of the ISSR bands were polymorphic and only eight bands were present in all the five *Eleusine* species analysed by Salimath et al. (1995). The total number of bands produced by all analysed primers in the ten species ranged between 71 in *Diplotaxis virgata* and 135 in *D. muralis*. Two hundred and fifty bands (approximately 51%) were found to be species-specific. Likewise, Wolff and Morgan-Richards (1998) found that most polymorphic RAPD and ISSR fragments were subspecies-specific in *P. major*. Figure 1 shows the amplification patterns generated using primers UBC888 (a) and UBC891 (b) across the ten species with the product-mixing strategy.

The minimum and maximum number of fragments generated per primer in all the species studied were 16 (primer UBC868) and 70 (primer UBC890), respectively (Table 2), with an average of 41.2 fragments. The average number of fragments per primer was higher for the 5´ triple-anchored primers (63.3) than for those with 3´ double-anchored primers (28.7) and 3´ single-anchored primers (37.5). Though we did not carry out an extensive comparison between 3´ double- and single-anchored and 5´ triple-anchored primers using the same core repeat, similar to the studies of Zietkiewicz et al. (1994), Moreno et al. (1998) and Blair et al. (1999), our results indicate that 5´-anchored primers were more non-specific (lack selective nucleotides at the critical 3´ end) than the others, thus generating a higher number of fragments. With respect to the 3´-anchored primers, because of the

**Table 2** List of ISSR primer sequences used, together with the scorable and size range of fragments resulting from each primer

| Primer | | No.of fragments scored | Aprox. fragment size range (bp) |
|---|---|---|---|
| Code | Sequence[a] | | |
| UBC811 | $(GA)_8C$ | 47 | 1250–300 |
| UBC818 | $(CA)_8G$ | 28 | 1300–320 |
| UBC841 | $(GA)_8YC$ | 40 | 1140–250 |
| UBC848 | $(CA)_8RG$ | 19 | 1125–420 |
| UBC857 | $(AC)_8YG$ | 27 | 1350–330 |
| UBC868 | $(GAA)_6$ | 16 | 900–230 |
| UBC878 | $(GGAT)_4$ | 39 | 1090–260 |
| UBC880 | $(GGAGA)_3$ | 25 | 1400–300 |
| UBC888 | $BDB(CA)_7$ | 57 | 1260–250 |
| UBC889 | $DBD(AC)_7$ | 66 | 1430–260 |
| UBC890 | $VHV(GT)_7$ | 70 | 1350–240 |
| UBC891 | $HVH(TG)_7$ | 60 | 1500–240 |

[a] R stands for puRine, Y for pYrimidine, B for non-A, D for non-C, H for non-G, V for non-T residues

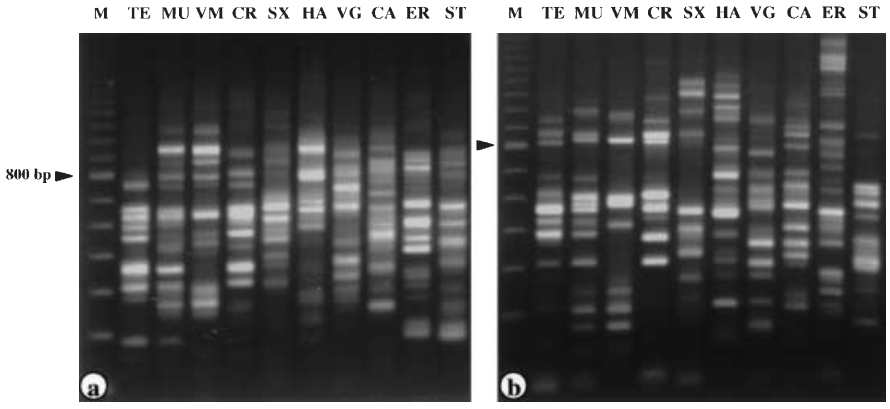


**Fig. 1** Inter-simple sequence repeat (ISSR) banding profiles obtained on 2% agarose gels for the ten *Diplotaxis* species (using the product-mixing strategy) with the primers UBC888 (**a**) and UBC891 (**b**). TE=*D. tenuifolia*, MU=*D. muralis*, VM=*D. viminea*, CR=*D. cretacea*, SX=*D. simplex*, HA=*D. harra*, VG=*D. virgata*, CA=*D. catholica*, ER=*D. erucoides*, ST=*D. siettiana*. M=molecular size marker (100-bp ladder, Pharmacia)

more restrictive conditions we have obtained a lower number of fragments in the double- than in the single-anchored ones. These results may be useful for a preliminary selection of ISSR primers for other plant species.

Reproducibility is essential for a technique to be used in fingerprinting. In the current experiment, we tested the repeatability of ISSR markers by performing separate PCR runs. The amplification of these markers was consistent and no new differences were detected in different experiments. Only very faint fragments were not reproducible and, as a consequence, such fragments were not scored in this study. This indicates that the ISSR approach is highly reproducible, which may be due to the use of longer primers and higher annealing temperatures (Nagaoka and Ogihara 1997; Moreno et al. 1998) compared with those used normally for other DNA-amplification-based techniques, such as the RAPD procedure which shows problems of reliability and repeatability (Ellsworth et al. 1993).

Upon comparing the template- and product-mixing banding profiles obtained for all ten species with the 12 ISSR primers, only small differences were found in the intensity of 20 bands; in addition, the template-mixing patterns generated six new bands that were not observed in the product-mixing patterns nor in any of the corresponding individuals separately. A similar behaviour has been observed by Davis et al. (1995), when they carried out DNA mixing experiments (with template- as well as product-mixing) to analyse RAPD markers. As in our experiments, they were able to generate new non-parental bands by PCR template-mixing experiments (mixing parental template DNAs in equal proportions prior to PCR). A possible explanation for the presence of the new bands in the template-mixing profiles may be the phenomenon of artifactual, heteroduplex band formation, which has been described by Ayliffe et al. (1994) for RAPD markers. During PCR reactions there are conditions causing denaturation/renaturation of co-amplified products from different mixed individual DNAs. Renaturation of intra-individual homoduplex molecules is accompanied by the formation of different possible inter-individual heteroduplex molecules. These heteroduplexes, involving complementary inter-individual strands with some differences in their sequence, would have abnormal secondary structures, and therefore a probable alteration of their electrophoretic mobilities compared to intra-individual homoduplexes. Thus, the six new bands in template-mixing patterns may be artifacts, in that they can not consist of discrete PCR products.

Because of these results it was decided to analyse the product-mixing banding profiles as representative of the different species studied. However, taking into account that in this study we have analysed 494 bands, and that only six artifactuals bands were generated with the template-mixing strategy, we propose that in such cases as this it would be more simple to use a template-mixing strategy, which is a less labour-intensive procedure than the product-mixing strategy by reducing the need for individual genomes (Furman et al. 1997).

## Genetic relationships among *Diplotaxis* species

The ISSR marker approach is particularly valuable in the study of the genus *Diplotaxis*, where extensive genetic characterisations of the nuclear genomes are lacking. We employed the ISSR technique in the analysis of ten *Diplotaxis* species to determine their genetic relatedness. Our results indicated that these species could be easily characterised by ISSR markers. The estimated similarity coefficients (based on simple matching values) among the ten *Diplotaxis* species ranged between 0.866 (*D. tenuifolia*/*Diplotaxis cretacea*) and 0.549 (*D. erucoides*/*D. muralis*) (Table 3). The dendrogram obtained by the UPGMA method using the 494 ISSR markers scored in the ten species clearly shows two defined groups, I and II, in Fig. 2. The Mantel test revealed a good and significant co-phenetic correlation ($r$=0.8; $P$=0.0005), which indicates a good fit to the cluster analysis (Rohlf 1992).

Group I from the dendrogram contains the species with gametic numbers of n=11 (*D. tenuifolia*, *D. cretacea* and *Diplotaxis simplex*), n=10 (*D. viminea*) and n=21 for the putative amphidiploid *D. muralis* derived from *D. tenuifolia* and *D. viminea* (Harberd and McArthur 1972). Table 3 shows higher similarity values among these group of species, especially between the pairs *D. tenuifolia/D. cretacea*, *D. viminea/D. muralis* and *D. tenuifolia/D. muralis*. This is due to the fact that

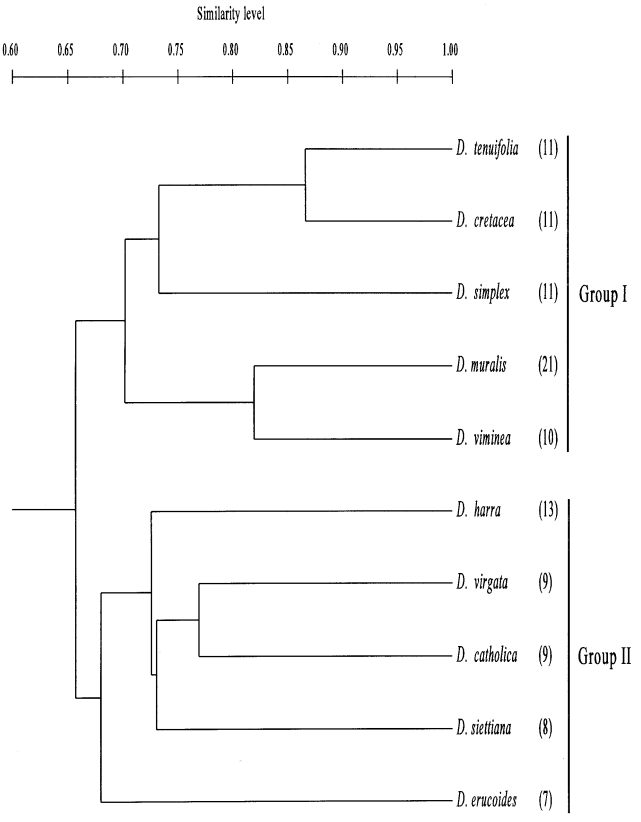


**Fig. 2** Dendrogram derived from a UPGMA cluster analysis, using the simple matching coefficient based on 494 ISSR bands, showing the relatedness among ten *Diplotaxis* species. In *brackets* is indicated the gametic number of each species

these taxa share a considerable number of markers (71 between *D. tenuifolia/D. cretacea*, 74 between *D. viminea/D. muralis*, and 64 between *D. tenuifolia/D. muralis*), indicating that these species form a close genetic assemblage within the genus *Diplotaxis*. The dendrogram shows that *D. tenuifolia* and *D. cretacea* seem to be the nearest, at a similarity level of 0.866; the other n=11 species, *D. simplex*, is joined to this previous cluster at a level of 0.733 (Fig. 2). This clustering is consistent with both cytological and morphological traits (Harberd 1972; Martínez-Laborde 1991a). In the other branch, *D. viminea* and the amphidiploid *D. muralis* also exhibit a high value of similarity (0.820), which seems reasonable since the former is the female parent of the amphidiploid, as has been deduced by the polypeptide composition of Rubisco subunits (Mummenhoff et al. 1993) and by using cpDNA markers (Warwick et al. 1992). The other putative partner, *D. tenuifolia*, also manifests a high similarity (0.779) to the amphidiploid (Table 3).

Different evidence has also demonstrated that these five species from group I (*D. tenuifolia*, *D. cretacea*, *D. simplex*. *D. viminea* and *D. muralis*) constitute a group of closely related taxa. From a morphological point of view, these taxa were considered as an infrageneric group – Section *Anocarpum* DC. — by Prantl (1891), and currently has been elevated to the rank of subgenus

**Table 3** Similarity matrix among ten *Diplotaxis* species using the simple matching coefficient based on 494 ISSR fragments scored

| Species | *D. tenuifolia* | *D. muralis* | *D. viminea* | *D. cretacea* | *D. simplex* | *D. harra* | *D. virgata* | *D. catholica* | *D. erucoides* | *D. siettiana* |
|---|---|---|---|---|---|---|---|---|---|---|
| *D. tenuifolia* | 1.000 | | | | | | | | | |
| *D. muralis* | 0.779 | 1.000 | | | | | | | | |
| *D. viminea* | 0.672 | 0.820 | 1.000 | | | | | | | |
| *D. cretacea* | 0.866 | 0.747 | 0.652 | 1.000 | | | | | | |
| *D. simplex* | 0.727 | 0.672 | 0.690 | 0.739 | 1.000 | | | | | |
| *D. harra* | 0.684 | 0.630 | 0.652 | 0.680 | 0.706 | 1.000 | | | | |
| *D. virgata* | 0.674 | 0.623 | 0.674 | 0.686 | 0.709 | 0.723 | 1.000 | | | |
| *D. catholica* | 0.686 | 0.636 | 0.686 | 0.678 | 0.725 | 0.727 | 0.769 | 1.000 | | |
| *D. erucoides* | 0.603 | 0.549 | 0.632 | 0.603 | 0.638 | 0.672 | 0.706 | 0.678 | 1.000 | |
| *D. siettiana* | 0.664 | 0.613 | 0.660 | 0.660 | 0.686 | 0.729 | 0.727 | 0.735 | 0.664 | 1.000 |

**Table 4** Comparison among taxonomic classification of the studied *Diplotaxis* taxa and the clusters obtained with ISSR-PCR data

| Taxonomical classification | | | Clusters grouped in the present study |
|---|---|---|---|
| Prantl (1891) | Schulz (1919) | Martínez-Laborde (1998)[a] | |
| Section *Anocarpum* | Section *Anocarpum* | Subgenus *Diplotaxis* | Group I |
| *D. tenuifolia, D. simplex, D. viminea, D.muralis* | *D. simplex D. viminea, D. muralis* | *D. tenuifolia, D. cretacea, D. simplex, D. viminea, D. muralis* | *D. tenuifolia, D. cretacea, D. simplex, D. viminea, D. muralis* |
| | | Subgenus *Rynchocarpum* | Group II |
| Section *Rynchocarpum* | Section *Rynchocarpum* | Sect. *Rynchocarpum* | |
| *D. virgata, D. catholica, D. erucoides* | *D. virgata, D. catholica, D. erucoides* | *D. virgata, D. catholica* Sect. *Heterocarpum* *D. siettiana* Sect. *Heteropetalum* *D. erucoides* | *D. virgata, D. catholica, D. siettiana, D. harra, D. erucoides* |
| Section *Catocarpum* | Section *Catocarpum* | Subgenus *Hesperidium* | |
| *D. harra* | *D. harra, D. tenuifolia, D. cretacea* | *D. harra* | |

[a] In Gómez-Campo and Martínez-Laborde (1998)

by Martínez-Laborde (in Gómez-Campo and Martínez-Laborde 1998) (see Table 4). Cytological data and studies based on the crossabilitiy of taxa (Harberd 1972; Takahata and Hinata 1983) have also shown the high affinity among these five species. Apart from morphological and cytological features, they share other characteristics with respect to chemical factors such as the bad odour due to the presence of glucosinolates. For example, allylglucosinolate is the principal component of *D. muralis* and *D. tenuifolia*, and high concentrations of 4-metylthiobutylglucosinolate are present in *D. viminea* (Al-Shehbaz 1985). Other studies with different methodologies have also revealed the close relationships existing among these five taxa including: flavonoid analyses (Sánchez-Yélamo and Martínez-Laborde 1991; Sánchez-Yélamo 1994), as well as seed proteins and seed isozyme electrophoretic patterns (Sánchez-Yélamo and Martínez-Laborde 1991; Sánchez-Yélamo et al. 1992), and other molecular analyses using cpDNA (Warwick et al. 1992) and cpDNA and mtDNA (Pradhan et al. 1992) [taking into account that the taxon *Diplotaxis pitardiana* analysed by these last authors is actually *D. simplex* due to a misidentification of the sample kept in the original germplasm bank (see Martínez-Laborde 1990).] The results obtained in the present study using nuclear DNA markers are congruent with all those previously mentioned, and support the above-mentioned assumption of Martínez-Laborde's (in Gómez-Campo and Martínez-Laborde 1998), reinforcing the taxonomic status of the group proposed by these and earlier authors.

Especially noteworthy is the case of *D. muralis*, with respect to its putative parents *D. tenuifolia* and *D. viminea* (Harberd and McArthur 1972). In the previously mentioned papers, the chromatographic flavonoid profiles of the amphidiploid, as well as the isozymatic and protein patterns, were always exactly a summation of the patterns of the putative parents (Sánchez-Yélamo and Martínez-Laborde 1991; Sánchez-Yélamo et al. 1992; Sánchez-Yélamo 1994); and the same was true when Mummenhoff et al. (1993) carried out a study of the polypeptide composition of Rubisco subunits. In the present paper using ISSR markers, the hypothesis of natural hybridization is again reinforced since the pattern exhibited by the amphidiploid, though it possesses its own bands, shares most of DNA fragments with one or both putative parents with respect to primers UBC888 and UBC891 (Fig. 1), and the same occurs for the other primers studied (data not shown).

Group II includes the cluster constituted by *D. virgata* and *Diplotaxis catholica* (both n=9), to which *Diplotaxis siettiana* (n=8) is joined, followed by *Diplotaxis harra* (n=13), while the last taxon of the group turns out to be the only n=7 species of the genus, *D. erucoides* (Fig. 2). In contrast to the species of group I, all the taxa of this second group share the characteristic of being odourless, probably due to the scarce amount of glucosinolates. *D. virgata* and *D. catholica* are related morphologically, and were included and maintained by different authors in the Section *Rynchocarpum* Prantl (Prantl 1891; Schulz 1919; Gómez-Campo and Martínez-Laborde 1998) (see Table 4). They constitute a cluster with a high genetic similarity (0.769; Table 3), to which *D. siettiana* is joined at a similarity level of 0.731 (Fig. 2). *D. siettiana* is an endemic taxon from the Spanish island Alborán, and originally has been subordinated to *D. catholica*, but morphological and chromosomal differences have resulted in its elevation to specific rank (Gómez-Campo 1981). The close relationships among these three taxa reflected in the dendrogram generated by ISSR markers are concordant with other molecular data obtained from cpDNA and mtDNA analyses (Pradhan et al. 1992), and support their specific status according to the most recent taxonomic classification (Gómez-Campo and Martínez-Laborde 1998): the three taxa belong to the subgenus

*Rynchocarpum*, but *D. virgata* and *D. catholica* are included in the Section *Rynchocarpum* and *D. siettiana* in the Section *Heterocarpum* (see Table 4).

*D. harra*, formerly placed by Schulz (1919) in the Section *Catocarpum* near to *D. tenuifolia*, demonstrates a great plasticity with respect to its association with other taxa of the genus depending on their biological characters, as has been demonstrated in different studies. For example, using cpDNA markers, Pradhan et al. (1992) and Warwick et al. (1992) found that *D. harra* was next to *D. tenuifolia*. However, when seed proteins or leaf flavonoids were analysed, this taxon appeared next to the *D. catholica* group (Sánchez-Yélamo et al. 1992; Sánchez-Yélamo 1994). All these results are surprising from a taxonomic point of view, since following the classification of Schulz (1919) *D. catholica* is included in the Section *Rynchocarpum*, and *D. harra* in the Section *Catocarpum*, or else into the subgenus *Hesperidium* according to the most recent classification of Martínez-Laborde (Gómez-Campo and Martínez Laborde 1998) (Table 4). Also, these species are recognised in separate cytodemes by Harberd (1972) and Takahata and Hinata (1983). Obviously, *D. harra* shares a very important part of its genic pool with other species of the genus. In the present work, using nuclear DNA markers, *D. harra* appears to be the closest to the group constituted by *D. catholica*, *D. virgata* and *D. siettiana*. This does not necessarily mean that it is related to them directly, but considering all the species studied, *D. harra* seems to be nearer to the Group-II species than to the Group-I species. As was indicated by Martínez-Laborde (1991b), this taxon shows a considerable amount of variation in several characters, even at the intrapopulation level, along its geographical distribution (from West Mediterranean region to Middle Orient), and has not been recognised as a different subspecies but rather belongs to a single species. However, in each one of the reports previously cited, different genotypes of this taxon have been used, which perhaps would have to be treated with the range of subspecies from a taxonomic point of view.

*D. erucoides* presents the lowest values of similarity with respect to the rest of species (Table 3), and this can be explained by the fact that this taxon is the one which presents the most distant genetic relationship compared to the others. This species, the only white-flowered one, possesses the lowest chromosome number of the genus and, as a consequence, we could hypothesise that *D. erucoides* would be related to a hypothetical ancestor involved in the origin of this group (or even of the genus), as it constitutes the taxon whose chromosome number is the smallest in a continuous series of haploid numbers ranging from 7 to 13 (with the exception of n=12, not found, and n=21 from *D. muralis*, due to its amphidiploid origin).

Though all the taxa included in the genus *Diplotaxis* have not been analysed here, the two groups defined in the ten species studied may be concordant with the idea suggested by several authors for a biphyletic origin of *Diplotaxis* (Gómez-Campo 1980; Warwick et al. 1992). In conclusion, we have demonstrated the high resolution in assessing relationships among species using the nuclear DNA marker ISSR-PCR. This approach clearly offers great potential for characterising related *Diplotaxis* species and can be applicable for similar use in other plant groups.

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## References

Al-Shehbaz IA (1985) The genera of *Brassiceae* (Cruciferae: Brassicaceae) in the southeastern United States. J Arnold Arbor Harv Univ 66:279–351

Ayliffe MA, Lawrence GJ, Ellis JG, Pryor AJ (1994) Heteroduplex molecules formed between allelic sequences cause nonparental RAPD bands. Nucleic Acids Res 22:1632–1636

Blair MW, Panaud O, McCouch SR (1999) Inter-simple sequence repeat (ISSR) amplification for analysis of microsatellite motif frequency and fingerprinting in rice (*Oryza sativa* L.). Theor Appl Genet 98:780–792

Charters YM, Robertson A, Wilkinson MJ, Ramsay G (1996) PCR analysis of oilseed rape cultivars (*Brassica napus* L. ssp. *oleifera*) using 5´-anchored simple sequence repeat (SSR) primers. Theor Appl Genet 92:442–447

Davis TM, Yu H, Haigis KM, McGowan PJ (1995) Template mixing: a method of enhancing detection and interpretation of codominant RAPD markers. Theor Appl Genet 91:582–588

Ellsworth DL, Rittenhouse KD, Honeycutt RL (1993) Artifactual variation in randomly amplified polymorphic DNA banding patterns. Biotechniques 14:214–217

Fang DQ, Roose ML, Krueger RR, Federici CT (1997) Fingerprinting trifoliate orange germ plasm accessions with isozymes, RFLPs, and inter-simple sequences repeat markers. Theor Appl Genet 95:211–219

Fang DQ, Krueger RR, Roose ML (1998) Phylogenetic relationships among selected *Citrus* germplasm accessions revealed by inter-simple sequence repeat (ISSR) markers. J Am Soc Hort Sci 123:612–617

Furman, BJ, Grattapaglia D, Dvorak WS, O'Malley DM (1997) Analysis of genetic relationships of Central American and Mexican pines using RAPD markers that distinguish species. Mol Ecol 6:321–331

Gilbert JE, Lewis RV, Wilkinson MJ, Caligari PDS (1999) Developing an appropriate strategy to assess genetic variability in plant germplasm collections. Theor Appl Genet 98:1125–1131

Gómez-Campo C (1980) Morphology and morphotaxonomy of the Tribe Brassiceae. In: Tsunoda S, Hinata K, Gómez-Campo C (eds) *Brassica* crops and wild allies. Japan Scientific Societies Press, Tokyo, pp 3–31

Gómez-Campo C (1981) Studies on Cruciferae. VIII. Nomenclatural adjustments in *Diplotaxis* DC. An Jardín Bot Madrid (CSIC) 38:29–35

Gómez-Campo C (1999) Taxonomy. In: Gómez-Campo C (ed) Biology of *Brassica* coenospecies. Elsevier Science, Amsterdam, pp 3–23

Gómez-Campo C, Martínez-Laborde JB (1998) Reajustes taxonómicos y nomenclaturales en la tribu *Brassiceae (Cruciferae)*. An Jardín Bot Madrid (CSIC) 56:379–381

Harberd DJ (1972) A contribution to the cyto-taxonomy of *Brassica* (Cruciferae) and its allies. Bot J Linn Soc 65:1–23

Harberd DJ, McArthur ED (1972) The chromosome constitution of *Diplotaxis muralis* (L.) DC. Watsonia 9:131–135

Hollingsworth ML, Hollingsworth PM, Jenkins GI, Bailey JP, Ferris C (1998) The use of molecular markers to study patterns of genotypic diversity in some invasive alien *Fallopia* spp. (Polygonaceae). Mol Ecol 7:1681–1691

Malik M, Vyas P, Rangaswamy NS, Shivanna KR (1999) Development of two new cytoplasmic male-sterile lines in *Brassica juncea* through wide hibridization. Plant Breed 118:75–78

Martínez-Laborde JB (1990) On previous misuses of the name *Diplotaxis pitardiana* Maire (Cruciferae: Brassicaceae). Taxon 39:117–118

Martínez-Laborde JB (1991a) Notes on the taxonomy of *Diplotaxis* DC. (Brassiceae). Bot J Linn Soc 106:67–71

Martínez-Laborde JB (1991b) *Diplotaxis harra* (Forsskal) Boiss. in Europe. Bot J Linn Soc 106:112–115

Moreno S, Martín JP, Ortiz JM (1998) Inter-simple sequence repeats PCR for characterization of closely related grapevine germplasm. Euphytica 101:117–125

Mummenhoff K, Eschmann-Grupe G, Zunk K (1993) Subunit polypeptide composition of Rubisco indicates *Diplotaxis viminea* as maternal parent species of amphiploid *Diplotaxis muralis*. Phytochemistry 34:429:431

Nagaoka T, Ogihara Y (1997) Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor Appl Genet 94:597–602

Pradhan AK, Prakash S, Mukhopadhyay A, Pental D (1992) Phylogeny of *Brassica* and allied genera based on variation in chloroplast and mitochondrial DNA patterns: molecular and taxonomic classifications are incongruous. Theor Appl Genet 85:331–340

Prantl K (1891**)** *Diplotaxis*. In: Engler A, Prantl K (eds) Die Natürlichen Pflanzenfamilien III. Wilhelm Engelmann, Leipzig, 2:176

Rao GU, Batra-Sarup V, Prakash S, Shivanna KR (1994) Development of a new cytoplasmic male-sterile system in *Brassica juncea* through wide hybridization. Plant Breed 112:171–174

Rohlf FJ (1992) NTSYS-PC: Numerical taxonomy and multivariate analysis system. Version 1.60. Exeter Software, New York

Salimath SS, de Oliveira AC, Godwin ID, Bennetzen JL (1995) Assessment of genome origins and genetic diversity in the genus *Eleusine* with DNA markers. Genome 38:757–763

Sánchez-Yélamo MD (1994) A chemosystematic survey of flavonoids in the Brassicinae: *Diplotaxis*. Bot J Linn Soc 115:9–18

Sánchez-Yélamo MD, Martínez-Laborde JB (1991) Chemotaxonomic approach to *Diplotaxis muralis* (Cruciferae: Brassiceae) and related species. Biochem Syst Ecol 19:477–482

Sánchez-Yélamo MD, Ortiz JM, Gogorcena Y (1992) Comparative electrophoretic studies of seed proteins in some species of the genera *Diplotaxis*, *Erucastrum* and *Brassica* (Cruciferae: Brassiceae). Taxon 41:477–483

Schulz OE (1919) *Diplotaxis*. In: Engler A (ed) Das Pflanzenreich IV. Wilhelm Engelmann, Leipzig, 105:149–179

Takahata Y, Hinata K (1983) Studies on cytodemes in subtribe *Brassicinae* (Cruciferae). Tohoku J Agric Res 33:111–124

Torres AM, Weeden NF, Martín A (1993) Linkage among isozyme, RFLP and RAPD markers in *Vicia faba*. Theor Appl Genet 85:937–945

Virk PS, Ford-Lloyd BV, Jackson MT, Newbury HJ (1995) Use of RAPD for the study of diversity within plant germplasm collections. Heredity 74:170–179

Wang Z, Weber JL, Zhong G, Tanksley SD (1994) Survey of plant short-tandem DNA repeats. Theor Appl Genet 88:1–6

Warwick SI, Black LD, Aguinagalde I (1992) Molecular systematics of *Brassica* and allied genera (Subtribe Brassicinae, Brassiceae) – chloroplast DNA variation in the genus *Diplotaxis*. Theor Appl Genet 83:839–850

Wolfe AD, Liston A (1998) Contributions of PCR-based methods to plant systematics and evolutionary biology. In: Soltis DE, Soltis PS, Doyle JJ (eds) Plant molecular systematics II. Chapman Hall, New York, pp 43–86

Wolfe AD, Xiang Q-Y, Kephart SR (1998) Assessing hybridization in natural populations of *Penstemon* (Scrophulariaceae) using hypervariable inter-simple sequence repeat (ISSR) bands. Mol Ecol 7:1107–1125

Wolff K, Morgan-Richards M (1998) PCR markers distinguish *Plantago major* subspecies. Theor Appl Genet 96:282–286

Wu K-S, Jones R, Danneberger L, Scolnik PA (1994) Detection of microsatellite polymorphisms without cloning. Nucleic Acids Res 22:3257–3258

Ziekiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20:176–183