Relationships among Morphological Characters, Isozymes Polymorphism and DNA Variability – the Impact on *Lactuca* Germplasm Taxonomy

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Abstract: Fifty one accessions of nineteen *Lactuca* species, the hybrid *L. serriola* × *L. sativa* and the related species *Mycelis muralis* were evaluated for morphological variability, esterase (EST) polymorphism, Amplified Fragment Length Polymorphism (AFLP) and relative DNA content. Sixteen *Lactuca* accessions were classified taxonomically on the basis of morphology, isozyme analysis and AFLP. Twenty-eight bands (isoforms) of EST were recorded allowing 82% of accessions to be distinguished. The relative DNA content, measured using flow-cytometry (DAPI staining), ranged from 2.02 pg in *L. capensis* to 17.96 pg in *L. canadensis*. The results from AFLP analysis and the relative DNA content measurement corresponded well with recent taxonomic classification of the genus *Lactuca*.

Keywords: AFLP; esterase; flow-cytometry; genetic resources; *Lactuca* spp.; morphology; PAGE electrophoresis; wild lettuce species; relative DNA content; taxomomy

Germplasm conservation has become a focus for many scientists throughout the world because of its underpinning role for plant breeding and global food production (Guarino *et al*. 1995; Gass *et al*. 1999), and the realisation of the potential value of genetic diversity for future generations (Huenneke 1991). Plant species maintained in genebanks (genetic resources) are characterised to enhance the understanding of the collections for both collection management and to potential users. The recording of classical morphological features (keystones of descriptive databases) has been complemented with records on biochemical and molecular features (Waycott & Fort 1994; Ayad *et al*. 1995), the data from which have become part of evaluation databases. The incorrect determination of taxonomic identity for some accessions has led to incorrect passport data being maintained in world genebanks (Lebeda *et al*. 1999, 2001; Doležalová *et al*. 2002b). The absence of or poor quality data on taxonomy and morphology can result in misinterpretation when using material for scientific purposes. Moreover, the paucity of data may limit the elucidation of relationships between individual accessions (species) within a given taxonomic group. This study, as a part

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of a broadly focused research on *Lactuca* genetic resources (Lebeda *et al*. 1999, 2001), addresses the theme of the precise taxonomic determination of individual accessions using classical methods and DNA analysis.

MATERIAL AND METHODS

Plant material. Fifty-one accessions of nineteen *Lactuca* species, a hybrid (*L. serriola* × *L. sativa*) and *Mycelis muralis* were studied. The majority of accessions represented either European or Asian species (*L. aculeata*, *L. altaica*, *L. indica*, *L. taraxacifolia*). Three species studied originated from South Africa (*L. capensis*, *L. dregeana*, *L. longespicata* or *L. schweinfurthii*) and two from North America (*L. biennis*, *L. canadensis*) (Doležalová *et al*. 2002b). These accessions are maintained currently at the Gene Bank in Olomouc (Research Institute of Crop Production – RICP, Praha-Ruzyně, Czech Republic). A majority of the experimental material was obtained from germplasm collections (Centre for Plant Breeding and Reproduction Research – CPRO-DLO, Centre for Genetic Resources – CGN, Wageningen, the Netherlands; Horticulture Research International – HRI, Gene Bank Unit, Wellesbourne, United Kingdom; United States Department of Agriculture – USDA, Agricultural Research Station – ARS, Salinas – Sal, California; Pullman, Washington, USA).

Morphological assessment. Sixteen plants from each accession were cultivated in plastic pots (19 × 19 cm) in a glasshouse under controlled conditions (temperatures by day 18–30°C, by night 12–16°C). The biennial (*L. biennis*, *L. canadensis*, *L. virosa*) and perennial species (*L. viminea*, *L. perennis*, *L. tatarica*) were maintained during the winter at the basal rosette stage at temperatures of 5–7°C in a glasshouse. The plants were evaluated throughout the growing period using published morphological descriptors (Doležalová *et al*. 2002a, 2003). Taxonomic determination was carried out according to the literature (Jeffrey 1966, 1975; Feráková 1976, 1977; Dethier 1982; Iwatsuki *et al*. 1995; Stace 1997) and a comparative study with reference herbarium specimens from the Herbarium Vadense – WAG, Wageningen University, the Netherlands and the Herbarium of the Faculty of Sciences of Komenský University in Bratislava – SLO, Slovakia. Voucher specimens of all accessions were deposited in the Herbarium of the Department of Botany of Palacký University – OL at Olomouc, Czech Republic.

Isozyme analysis. Three young basal rosette leaves were taken (4–5 weeks after transplanting) from 3 plants per accession. A mixed sample of all leaves per accession was used for analysis. Extraction was carried out according to Aung and Evans (1987). Samples were separated by electrophoresis (30 mA, 390 V, 2 h, 4°C) following the method of Laemmli (1970) with a discontinuous buffer system using a Standard Vertical Gel Electrophoresis Unit (Sigma) and EC3000P power supply (E-C Apparatus Corporation). PAGE gels were stained specifically for esterase (EST) (Manchenko 1994).

AFLP (Amplified Fragment Length Polymorphism). Fresh primary leaves were collected from each plant. The DNA extraction was performed according to Vosman *et al*. (1992). The AFLP procedure was performed according to Vos *et al*. (1995). The final restriction fragment amplification was performed using two primer combinations: E35/M48 and E35/M49.

Data analysis. AFLP fragments were scored as present/absent. The dendrogram was constructed using UPGMA (Unweighted Pair Group Method Average). The clustering method and similarity coefficient were tested using the procedure NCSS 97 (Statistical Solutions Ltd, Cork, Irish).

Relative DNA content. Young basal rosette leaves from 4–5 week old plants were used for DNA content estimation. Three leaves per accession were pooled and analysed. The measurement of each accession sample was repeated four times. The analysis was performed using a flow-cytometer (Partec GmbH, Germany). The linearity of the instrument was adjusted using trout erythrocytes for DAPI staining (Doležel *et al*. 1989). *Lactuca sativa* cv. British Hilde (absolute DNA content of 5.5 pg – PI = propidium iodide) was used as an external standard. Calibration of the flow-cytometer was based on measurements of 30 individuals (four replicates) of *L. sativa* cv. British Hilde.

Approximately 20 mg of fresh leaf tissue was collected in 800 μl buffer OTTO I (Otto 1990), subsequently 180 μl of buffer OTTO II (Otto 1990) containing DAPI (diamophenylindole) was added. The suspension was filtered through nylon mesh (40 μm pore size) and examined immediately. At least 3000 nuclei per sample were measured and the relative DNA content of a test sample was calculated as follows:

2C relative DNA content = (Sample peak mean/Standard peak mean) × 2C relative DNA content of standard (5.5 pg)

One-way ANOVA analysis and Scheffe's test were carried out using the Program NCSS 97. A dendrogram of relative DNA content similarity of the individual *Lactuca* accessions was constructed using the unweighted pair group method average (UPGMA) clustering procedure.

RESULTS AND DISCUSSION

Morphological assessment, isozyme variability & AFLP. The comparison of the data from the morphological study (e.g. stem, leaves, inflorescence, ligules and achenes) of individual *Lactuca* spp. accessions with reference herbarium specimens demonstrated that 16 accessions were incorrectly identified taxonomically (Table 1). This result was confirmed by the isozyme analysis (EST). Twenty eight bands (isoforms) of EST were recorded allowing 82% of the accessions to be distinguished. Zymograms of EST isozyme spectra of some critical accessions are presented in Figure 1. The results of

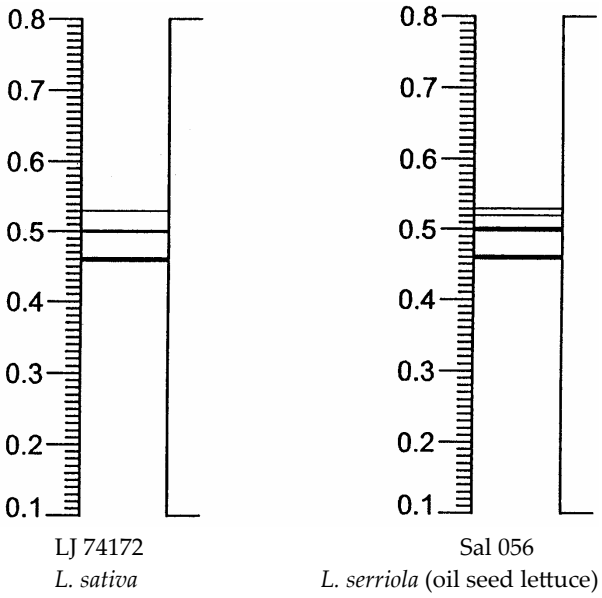


Figure 1. Comparison of *L. sativa* and *L. serriola* EST zymograms

Table 1. Taxonomic redetermination of *Lactuca* genetic resources accession based on morphology, esterase polymorphism, AFLP and relative DNA content analysis

Original determination	Accession number – international or EVIGEZ	Redetermination
Cicerbita alpina Wallr., syn. *L. alpina* Benth. et Hook. f.	09-H58-0123	*L. serriola* L.
Ixeris dentata Nakai, syn. *L. dentata* C.B. Robinson	PI 234204	*L. serriola* L. (oilseed lettuce)
L. altaica Fisch. et C.A. Mey.	LJ 74172	*L. sativa* L. (primitive form)
	LJ 74179	*L. sativa* L. (primitive form)
	Sal 056	*L. serriola* L. (oilseed lettuce)
L. angustana All.	Sal 059	*L. sativa* L. var. *angustana* Irish
L. homblei De Wild.	CGN 11322	probably *L. schweinfurthii* Oliver et Hiern or *L. longespicata* Wildem.
L. indica L., syn. *L. squarrosa* Miq.	PI 236396	*L. serriola* L. (oilseed lettuce)
L. livida Boiss. et Reut.	LJ 75009	*Lactuca* sp.
L. livida Boiss. et Reut.	LJ 75150	*Lactuca* sp.
L. livida Boiss. et Reut.	Sal 069	*L. dregeana* DC.
L. livida Boiss. et Reut.	Sal 070	*L. dregeana* DC.
L. quercina L.	Sal 073	*L. sativa* L. (oak leaf lettuce)
L. quercina L.	CGN 05808	*L. sativa* L. (primitive form)
L. serriola f. *integrifolia* (S.F. Gray) S.D. Prince et R.N. Carter	09-H58-0113	*L. serriola* L. f. *serriola*
Youngia denticulata (Houtt.) Kitamura syn. *L. denticulata* Maxim.	PI 234204	*L. serriola* L. (oilseed lettuce)

clustering analysis based on AFLP data are shown in Figure 2.

The majority of the 16 accessions were reclassified to the species *L. serriola*. The accessions identified with incorrect passport data were: *Cicerbita alpina* Wallr. (syn. *L. alpina* Benth. et Hook. f.); Sal 056 determined as *L. altaica* Fisch. et C.A. Mey.; PI 234204 *Ixeris dentata* Nakai (syn. *L. dentata* C.B. Robinson); *Youngia denticulata* (Houtt.) Kitamura (syn. *L. denticulata* Maxim.); PI 236396 *L. indica* L. (syn. *L. squarrosa* Miq.) (Table 1). All of them showed morphological features characteristic for the species *L. serriola*. Accessions PI 234204 and PI 236396 belong to the group of oil seed lettuce with entire undivided rosette and cauline leaves, and with large plump seeds. Boukema *et al*. (1990) reported that oil seed lettuce was related either to *L. serriola* or *L. sativa*, and recorded the existence of transitional types between these species. EST zymograms for these accessions showed the same spectrum as *L. serriola*. This conclusion was confirmed by AFLP analysis. Based on the morphology of rosette leaves the accession of *L. serriola* f. *integrifolia* was reclassified as *L. serriola* f. *serriola*.

The second largest group of reclassified accessions belongs to the species *L. sativa* (Table 1). Accessions LJ 74172 and LJ 74179 originally determined as *L. altaica* Fisch. et C.A. Mey. were classified as primitive forms of *L. sativa*. Plants of these accessions exhibited rosettes of densely accumulated, fresh green leaves that did not form heads, as reported by Ryder and Whitaker (1976). In addition, trichomes were recorded on the midrib of cauline leaves, thus supporting the classification as primitive forms of *L. sativa*. Isozyme analysis (Lebeda *et al*. 2001) and AFLP data supported the reclassification of these accessions. On the AFLP dendrogram these 2 accessions form a clearly separate group with the accession Sal 056 (Figure 2), the latter having morphological features closest to *L. serriola* (Harlan 1986). However the EST and AFLP analyses showed close links to *L. sativa*. Two accessions Sal 073 and CGN 05808 originally determined as *L. quercina* were redetermined as primitive forms of *L. sativa* also. They were characterised by fresh green leaves, which did not form heads, and serriola-like seeds rather than the black characteristic seed of *L. quercina* (Hegi 1987). Accession Sal 073 belongs to the group of oak leaf lettuces with anthocyanin coloration of the leaves as reported by Vries and Raamsdonk (1994). *L. angustana* (Sal 059) was classified as *L. sativa* L. var. *angustana* Irish (Table 1).

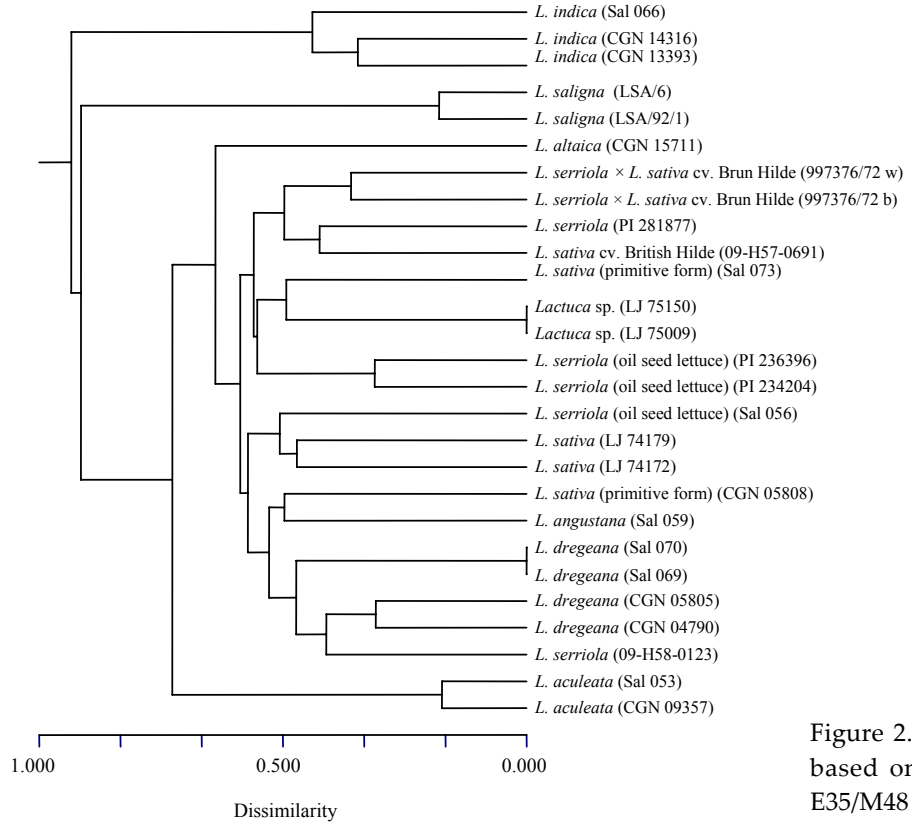


Figure 2. Dendrogram of *Lactuca* spp. based on AFLP polymorphism with E35/M48 primers combination

The morphology and life cycle of the accessions Sal 069 and Sal 070, originally determined as *L. livida* Boiss et Reut., did not correspond with the literature data (Feráková 1977; Velasco Negueruela 1981) and were classified as *L. dregeana* DC. The EST zymogram banding patterns (Lebeda *et al*. 2001) and similar relative DNA contents (Doležalová *et al*. 2002b) confirmed this conclusion. Also the AFLP analysis demonstrated a close relationship between Sal 069 and Sal 070 and taxonomically validated specimens of *L. dregeana* (Figure 2).

The morphology of accessions LJ 75009 and LJ 75150, previously determined as a *L. livida*, did not correspond with the botanical description of this species in local floras (Feráková 1977; Rollán 1985). The generic name *Lactuca* sp. (Table 1) has been given to these accessions in the absence of passport data and available reference herbarium material.

The accession maintained as *L. homblei* De Wild. (CGN 11322) (Table 1) is more likely to be *L. schweinfurtii* Oliv. & Hiern or *L. longespicata* De Wild. as described by Jeffrey (1966). Since these Central African species are not available in gene bank collections, there is a need for the development of reference herbarium specimens.

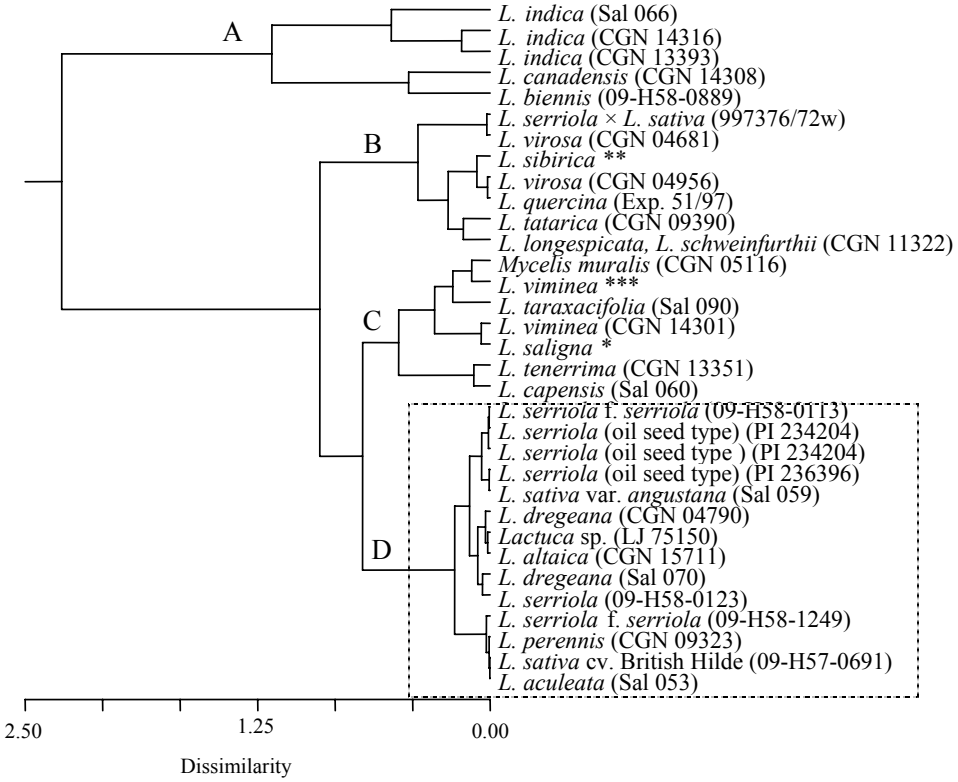
Relative DNA content measurement. Variability in the relative DNA content was found in the accessions studied (Table 2). The highest relative DNA content was observed in North American species *L. biennis* and *L. canadensis* (16.34 and 17.96 pg). The relative DNA content in 3 accessions of *L. indica* ranged from 11.87 to 14.12 pg. In contrast *L. capensis* (2.02 pg) and *L. tenerrima* (2.35 pg) had the smallest relative DNA contents. The remaining accession/taxa formed 3 definable groups. A group with relative DNA content ranging from 3.07 pg in *L. taraxacifolia* to 4.76 pg in *L. saligna* consisted of *Mycelis muralis*, *L. viminea*, *L. taraxacifolia* and *L. saligna*. The majority of species (both forms of *L. serriola*, *L. aculeata*, *L. altaica*, *L. angustana*, *L. dregeana*, *L. livida*, *L. sativa*, *L. perennis*) had a relative DNA content in the range from 5.49 pg (*L. aculeata*) to 6.54 pg (L. *angustana*). The remaining group with a slightly higher relative DNA content (7.51 to 9.75 pg) included *L. longespicata* or *L. schweinfurthii*, *L. sibirica*, *L. tatarica*, *L. quercina*, *L. virosa* and the hybrid *L. serriola* × *L. sativa*.

Overall the cluster analysis based on relative DNA content revealed that accessions could be classified in four main groups A, B, C, D (Figure 3). The group of accessions with the highest relative DNA content (A) consisted of North American species *L. biennis*, *L. canadensis* as well as the 3 accessions of *L. indica*. There are 2 subgroups in group C, *L. capensis* and *L. tenerrima* with the smallest relative DNA contents, and are clearly separated from the subgroup with *Mycelis muralis*, *L. viminea*, *L. taraxacifolia* and *L. saligna*. Group B with slightly higher relative DNA content formed two parallel clusters containing the species *L. virosa* and the hybrid *L. serriola* × *L. sativa*; *L. sibirica*, *L. quercina*, *L. tatarica* and *L. longespicata* or *L. schweinfurthii*. Three parallel clusters could be distinguished within a group with medium relative DNA content (D): the first cluster, *L. serriola* f. *serriola*, *L. sativa*, *L. angustana*; the second cluster, *L. dregeana*, *Lactuca* sp., *L. altaica*, *L. serriola*; the third cluster, *L. serriola* f. *serriola*, *L. perennis*, *L. sativa* cv. British Hilde and *L. aculeata*.

Data on the relative DNA contents complement the information from studies on morphology and polymorphism of EST (Lebeda *et al*. 1999, 2001). In a study on vascular plants Bennett and Leitch (1997) showed that the genus *Lactuca* belongs to a group of species with a medium genome size. The different relative DNA amounts were measured in the three most common species *L. serriola*, *L. saligna* and *L. virosa*. The highest value of relative DNA

Table 2. Relative DNA content established using flow-cytometry in selected *Lactuca* genetic resources (arranged according to Doležalová *et al*. 2002b)

2C relative DNA content	Group	*Lactuca* spp.
11.8–18.0	A	*L. biennis*, *L. canadensis*, *L. indica*
7.5–9.7	B	*L. schweinfurthii* or *L. longespicata*, *L. quercina*, *L. serriola* × *L. sativa*, *L. sibirica*, *L. tatarica*, *L. virosa*
5.5–6.5	D	*L. aculeata*, *L. altaica*, *L. dregeana*, *L. perennis*, *L. sativa* (primitive form), *L. sativa* var. *angustana*, *L. sativa* cv. British Hilde, *L. serriola* f. *serriola*, *L. serriola* (oil seed type)
2.0–4.8	C	*L. capensis*, *L. saligna*, *L. taraxacifolia*, *L. tenerrima*, *L. viminea*, *Mycelis muralis*



L. saligna * bulk of accessions LSA/92/1, LSA/6, PI261653
L. sibirica ** bulk of accessions W 9516, W 9520, W 9523
L. viminea *** bulk of accessions LVIM98/2, 7139, 7140

Figure 3. Cluster analysis of wild *Lactuca* spp. based on data of relative DNA content measurement (arranged according to Doležalová *et al*. 2002b)

content was observed in *L. virosa* accessions (7.51 pg and 8.76 pg) and the smallest in *L. saligna* (4.76 pg). The relative DNA contents in all the *L. serriola* accessions ranged from 5.58 to 6.52 pg. These data are in accordance with that of Koopman and De Jong (1996) and Koopman (1999, 2000). *L. serriola*, *L. sativa* (oil seed type) and *L. angustana* are classified in the largest cluster (D) in Figure 3.

Lindqvist (1960) considered the species *L. angustana* as a variety of *L. serriola*, while Feráková (1977) treated *L. angustana* as a form of *L. serriola*. The position of *L. angustana* on the cluster-analysis of DNA content indicates a relationship to the species *L. serriola*, however morphological features and AFLP analysis (Figure 2) indicate a relationship to the species *L. sativa* (Table 1).

L. dregeana, *L. aculeata* and *L. altaica* (section *Lactuca*, subsection *Lactuca*) are species closely related to *L. sativa* (Zohary 1990) and are considered a part of its primary genepool. The relationship of these accessions and *L. serriola* is reflected in their classification in a common cluster (Figure 3). However, *L. perennis* also appeared in this cluster even though it belongs to the subsection *Cyanicae* (Feráková 1977). Koopman *et al*. (1998) proposed to exclude the subsection *Cyanicae* from the genus *Lactuca*.

Some of the Group B (Table 2) taxa, *L. tatarica*, *L. sibirica* (section *Mulgedium*), *L. quercina* (section *Lactucopsis*) and the African *L. longespicata* or *L. schweinfurthii* are rather distant from the section *Lactuca*. Significant differences between these species and a group of species with small relative DNA content were found by Doležalová *et al*. (2000b). Interestingly 2 species of section *Lactuca*, did not align closely with the other section *Lactuca* taxa, both accessions of *L. virosa* were present in group B, while *L. saligna* was in the group C with very low relative DNA content. These results are in accordance with the RFLP data of Kesseli *et al*.

(1991). However, the results conflict with those of Koopman *et al*. (1993) who reported a close relationship between *L. sativa*, *L. serriola* and *L. saligna*, while *L. virosa* was rather distant. The presence of *L. capensis* and *L. tenerrima* in a cluster of taxa with very small DNA content is in agreement with their low chromosome number, although also in this group are a species *Mycelis muralis*, *L. viminea* and *L. taraxacifolia* with $n = 9$.

The position of *L. biennis*, *L. canadensis* and the three accessions of *L. indica* in the cluster with very high relative DNA content supports Babcock's *et al*. (1937) hypothesis of a close relationship of the North American and Old World *Lactuca* species.

The results on relative DNA content and AFLP analysis confirmed close taxonomic relationship between species belonging to the section *Lactuca* as reported by Koopman *et al*. (1998) and more or less correspond with recent taxonomic classifications of the genus *Lactuca* and its division into sections and subsections (Feráková 1977; Lebeda & Astley 1999; Koopman *et al*. 2001). These methods together with the EST analysis proved to be complementary methods in the elucidation of taxonomic relationships. The detailed morphological study and the comparison with reference herbarium specimens provided the basis for the precise taxonomic reclassification of taxa.

Thus only complex research, which includes classical taxonomic studies in tandem with a molecular approach, can solve these complex taxonomic questions objectively.

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Abstrakt

Doležalová I., Lebeda A., Dziechciarková M., Křístková E., Astley D., van de Wiel C.C.M. (2003): **Vztahy mezi morfologickými znaky, polymorfismem isoenzymů a variabilitou DNA – význam v taxonomii genových zdrojů rodu** *Lactuca*. Czech J. Genet. Plant Breed., **39**: 51–59.

U 51 položek genových zdrojů 19 druhů rodu *Lactuca* (locika), kříženec *L. serriola* × *L. sativa* a příbuzného druhu *Mycelis muralis* byla zkoumána morfologická variabilita, polymorfismus esteras, AFLP (Amplified Fragment Length Polymorphism) a relativní obsah DNA. Na základě studia morfologie doplněného isoenzymovou analýzou a AFLP bylo 16 položek locik taxonomicky redeterminováno. U studovaného souboru bylo zaznamenáno 28 bendů (isoforem) esteras, pomocí nichž bylo možné rozlišit 82 % položek. Relativní obsah DNA stanovený flow-cytometrickou metodou (barvení DAPI) se pohyboval od 2,02 pg u *L. capensis* do 17,96 pg u *L. canadensis*. Výsledky získané analýzami AFLP a měřením relativního obsahu DNA víceméně korespondují se současnou taxonomickou klasifikací rodu *Lactuca*.

Klíčová slova: AFLP; esterasy; průtoková cytometrie; genové zdroje; *Lactuca* spp.; morfologie; PAGE elektroforéza; plané druhy locik; relativní obsah DNA; taxonomie

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