A numerical analysis of karyotypes and DNA amounts in lettuce cultivars and species (*Lactuca* subsect. *Lactuca*, Compositae)

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SUMMARY

Karyotype and relative DNA content were used to characterize *Lactuca sativa*, *L. serriola*, *L. saligna* and *L. virosa* and to determine their evolutionary relationships. In these species karyotype analyses requiring the identification of the homologues are unreliable, because not all chromosomes can be distinguished by their length and centromere position, and no useful additional cytological markers are available. Therefore the karyotypes were established using numerical parameters describing the whole metaphase complement rather than the individual chromosomes, namely: intra- and interchromosomal asymmetry index, total chromosome length and area, and number of discernible satellites. The karyotype data were supplemented with data on relative DNA content. No significant differences were found between *L. sativa* and *L. serriola*, whereas *L. saligna* differed significantly from *L. sativa/serriola* only in its relative DNA amount. *L. virosa* differed from *L. saligna* and *L. sativa/serriola* for all parameters. The largest differences were found between *L. saligna* and *L. virosa*, although both have asymmetric karyotypes compared to *L. sativa/serriola*. Since asymmetric karyotypes in Compositae tribe Cichorieae (including *Lactuca*) are considered to be derived it follows that *L. saligna* and *L. virosa* are advanced species that evolved in different directions.

Key-words: Asteraceae, Compositae, karyotype analysis, *Lactuca*, lettuce.

INTRODUCTION

Ferakova (1977) proposed a subdivision of the west European *Lactuca* L. species into four sections: *Phaenixopus* (Cass.) Benth., *Mulgedium* (Cass.) C. B. Clarke, *Lactucopsis* (Schultz-Bip. ex Vis. et Panč.) Rouy and *Lactuca*. In the section *Lactuca* two subsections were recognized: *Lactuca* and *Cyanicae* DC. The subsection *Lactuca* comprises the species *L. serriola* L., *L. sativa* L. (cultivated lettuce), *L. saligna* L., *L. altaica* Fisch. et Mey., *L. virosa* L. and *L. livida* Boiss. et Reut. All species in the subsection *Lactuca* are diploids with 2n=18 chromosomes.

Since 1984 the species *L. serriola*, *L. sativa*, *L. saligna* and *L. virosa* have been the subject of a biosystematic study at the Department of Plant Taxonomy, Wageningen Agricultural University. The objectives of the study were to examine the species boundaries and to determine evolutionary relationships among the species. Karyotype studies and analyses of DNA sequences were part of this study.

Karyotype study is a useful tool in taxonomy either to characterize taxa or to reconstruct their phylogeny (see e.g. Stebbins 1971). Its value for phylogeny reconstruction in Compositae has been amply demonstrated by Babcock (1947) for *Crepis*. Lindqvist (1960) was the first to establish detailed *Lactuca* karyotypes from chromosome measurements. The karyotypes of *L. sativa* (six accessions) and *L. serriola* (eight accessions) were found to be identical for all cases, which was confirmed later by Chatterjee & Sharma (1969) and Haque & Godward (1985). The karyotype of *L. saligna* (two accessions) was found to be slightly different from that of *L. sativa/serriola* while distinct differences were observed between *L. virosa* (three accessions) and the other three species. Lindqvist also studied the shape and number of the microsatellites of the nucleolar organizing chromosomes and found one pair for *L. virosa* and two pairs for the other three species.

Lindqvist (1960) and Haque & Godward (1985) described the chromosome pairs on the basis of length, centromere position and presence of microsatellites. The values for chromosome lengths and arm ratios of putative homologues from different complements were averaged, assuming these to be characteristic for a particular chromosome pair in the karyogram. However, in the case of only slight differences among the non-homologues, chromosome length and arm ratio are unreliable parameters to identify chromosomes. Matérn & Simak (1968), Bentzer *et al.* (1971) and Fukui & Kakeda (1994) demonstrated that analyses based only on these parameters give rise to considerable numbers of misidentifications. Because misidentified chromosomes will not be properly ranked when ordered by length in a karyogram, Simak (1962) designated these misidentifications as 'reversal of order'. Misidentification of chromosome arms of metacentric chromosomes was designated as 'arm reversal'. Since the differences in lengths and arm ratio values of the subsequent chromosome pairs in the diploid chromosome sets of *Lactuca* are small, there is an actual risk of arm reversal and reversal of order. Consequently, karyograms as constructed by Lindqvist (1960) and Haque & Godward (1985) are unreliable.

The identification problem could be solved by the use of cytological markers such as C- and N-bands. However, the banding patterns of the individual *Lactuca* chromosomes were insufficiently different to enable identification of all chromosomes in the complement (Koopman *et al.* 1993). As yet, other cytological markers have not been tested for this purpose and therefore an alternative approach was chosen.

In this paper we applied a numerical analysis of the karyotypes of *L. sativa*, *L. serriola*, *L. saligna* and *L. virosa* using parameters for the total cell complement rather than for individual chromosomes. Thus, identification of homologues was no longer necessary and the risk of reversals was avoided. The parameters used describe the karyotype in terms of symmetry (intra- and interchromosomal asymmetry index) and amount of chromosome material (total chromosome length and total chromosome area). The karyotype data were supplemented with data on relative DNA content. Using these five parameters, 10 metaphase plates per accession were compared in a principal component analysis (PCA) and an analysis of variance followed by a Tukey HSD procedure. Based on the results, species boundaries and phylogenetic relationships of

Table 1. *Lactuca* accessions of the Centre for Genetic Resources, The Netherlands (CGN), used for karyotyping. The cultivar groups are according to Rodenburg (1960). *L. serriola*, *L. saligna* and *L. virosa* are wild species

Species	CGN accession no.	Cultivar name	Cultivar group
L. sativa	5979	Balady	Cos
L. sativa	4546	Celtuce	Stalk
L. sativa	4600	Great Lakes 65	Crisphead
L. sativa	4707	Oak Leaf	Cutting
L. sativa	5135	Saffier	Butterhead
L. sativa	4869	Tetue de Nimes	Latin
L. sativa	5208	Mataro Tres Ojos	Cos
L. serriola	10881		
L. saligna	5310		
L. virosa	9315		

the four species were discussed. Because the differences in visibility of the satellites among *L. sativa*, *L. serriola* and *L. saligna* were assumed to have no taxonomical significance, data on the satellites were excluded from the analyses.

MATERIALS AND METHODS

Plant material

Ten *Lactuca* accessions of the Centre for Genetic Resources, The Netherlands (CGN) collection, including the species *L. serriola*, *L. saligna*, *L. virosa* and six *L. sativa* cultivar groups (Rodenburg 1960) were used (Table 1). Voucher specimens of all accessions were deposited at the Herbarium Vadense (WAG), supplemented with photographs of the plants in rosette, bolting and flowering stage, and with pappus preparations and seed samples.

Young plantlets were grown in the greenhouse at 18/22°C. Actively growing root tips and young leaves were collected for chromosome preparations and DNA measurements, respectively.

Chromosome preparations

Root tips were collected between 0800 and 0900 h and pretreated in 1·5 mM 8-hydroxyquinoline for 2·25 h at 18°C for metaphase arrest and chromosome shortening. The material was fixed in acetic acid–ethanol 1:3 and stored at −20°C until use.

After carefully rinsing in deionized water root tips were hydrolyzed in 1 N HCl at 58°C for 6·25 min. Subsequently, the root meristems were rinsed again and squashed in a drop of acetic acid 45% on a glass slide. After freezing the slide in liquid nitrogen, the cover slip was removed and the slide was briefly rinsed in acetic acid–ethanol and ethanol 96% steps, respectively. The squash preparations were left to dry overnight, stained in 1% Giemsa in deionized water for 3 min, air-dried and mounted in Entellan-Neu (Merck, Darmstadt, Germany).

For each of the accessions a sample of 10 different plants was used for chromosome study. Only one metaphase complement per plant was selected showing well-spread chromosomes with distinctive centromeres, chromatids and satellites.

Chromosome measurements

Chromosomes were measured on enlarged prints at a final magnification of $c. \times 3200$ using a digitizing tablet connected to a PC. To minimize observation inaccuracies, both the short and the long arm lengths of every chromatid were measured three times and their values were averaged.

Three parameters were derived from the arm length data: (i) intrachromosomal and (ii) interchromosomal asymmetry index (Romero Zarco 1986) and (iii) total chromosome length in μm. The intrachromosomal asymmetry index A_1 equals (1-complement mean of the ratio of the short and long arm of each chromosome). The interchromosomal asymmetry index A_2 is the ratio of the standard deviation and the mean chromosome length for a complement.

Total chromosome area was estimated by computer imaging. The photo prints were recorded with a CCD camera and digitized by a DT-1451 framegrabber (Data Translation, Marlboro, USA). The final resolution was 0·054 μm/pixel in both directions (image size 512×712 pixels). The images were analysed using standard routines of the software package Scil-Image (TPD-TNO, Delft, The Netherlands).

DNA measurements

Relative DNA content of four plants of each accession was determined by Plant Cytometry Services (Schijndel, The Netherlands) using a method modified from De Laat & Blaas (1984). The analysis was performed with the ICP 22 (Ortho Diagnostic Systems, Beerse, Belgium) flow cytometer using *Lycopersicon esculentum* 'Tiny Tim' as internal reference. The relative DNA content of each sample was calculated by dividing the median value of the obtained DNA histogram of a *Lactuca* sample by that of the reference.

Statistics

Differences among the accessions regarding asymmetry indices, total chromosome length, total chromosome area and relative DNA content were tested for significance at the 5% level in a one-way analysis of variance followed by a Tukey-HSD procedure using SPSS/PC 4.0 (Norusis 1990). The NTSYS-PC program version 1.80 (Rohlf 1993) was used to perform a PCA on A_1, A_2, total chromosome length, total chromosome area and relative DNA content.

RESULTS

Figure 1 gives examples of metaphase complements of the four species. Note the satellite chromosome pairs 7/8 and 9/10 for *L. sativa*, 4/5 and 9/10 for *L. serriola*, 5/6 and 10/11 for *L. saligna* and 9/10 for *L. virosa*. The mean number of visible satellites per complement in *L. sativa* is 3·5 for 'Balady' and 'Tetue de Nimes', 3·8 for 'Celtuce', 'Great Lakes 65' and 'Oak Leaf', 3·9 for 'Saffier' and 4·0 for 'Mataro Tres Ojos'. In *L. serriola* the mean number of visible satellites per complement is 2·5 and in *L. saligna* 3·5. In *L. virosa* two satellites were visible in all cells. Figure 2 shows schematic representations of the metaphase plates of Fig. 1. The chromosomes are ordered in sequence of decreasing length. Reversals of order in the complements of *L. serriola* and *L. saligna* become obvious by the odd number of chromosomes between the satellite chromosome pairs in their karyograms (Fig. 2).
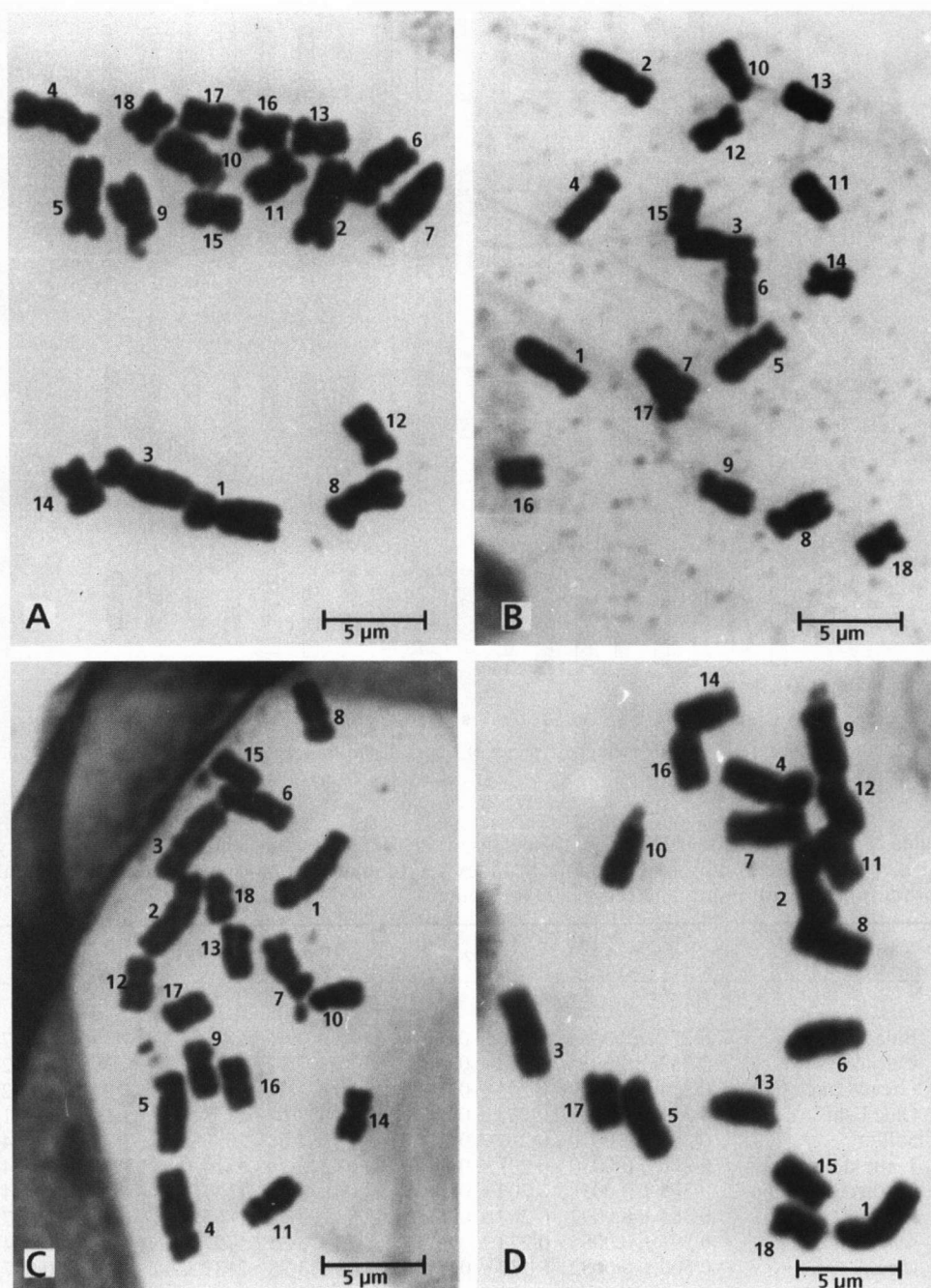
Fig. 1. Metaphase plates of *L. sativa* (A), *L. serriola* (B), *L. saligna* (C) and *L. virosa* (D).

Table 2 presents the data on asymmetry indices, total chromosome area, total chromosome length and relative DNA content of all accessions. The intrachromosomal asymmetry index A_1 of *L. virosa* is significantly higher than that of all other accessions.
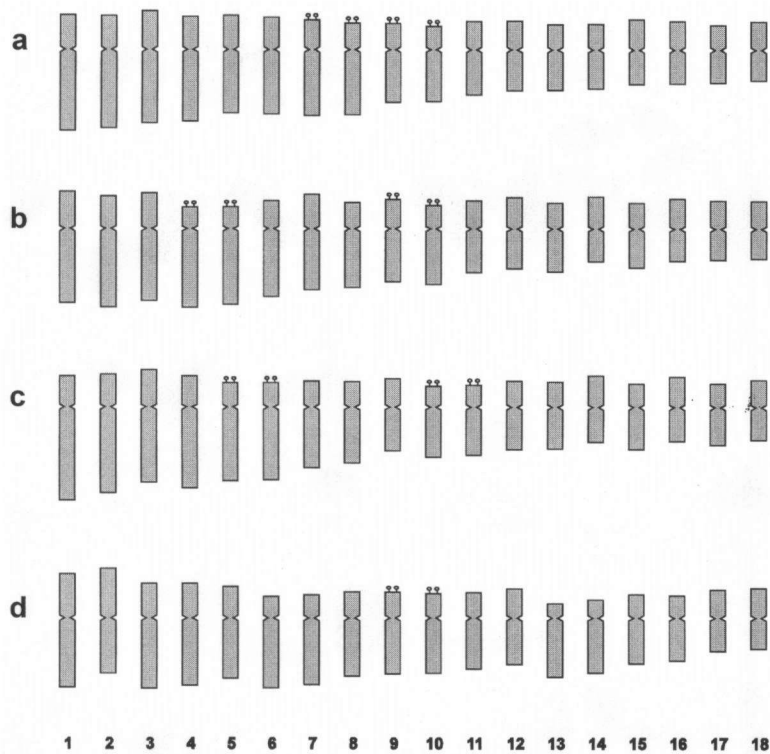
Fig. 2. Idiograms representing the metaphase plates of Fig. 1. *L. sativa* (a), *L. serriola* (b), *L. saligna* (c) and *L. virosa* (d).

Table 2. Means and standard errors of the means of the parameters intrachromosomal asymmetry index (A_1), interchromosomal asymmetry index (A_2), total chromosome area in μm^2, total chromosome length in μm and relative DNA content

Accession	A_1	A_2	Area	Length	DNA
L. sativa					
'Balady'	$0\cdot377 \pm 0\cdot0059$	$0\cdot203 \pm 0\cdot0064$	$63\cdot87 \pm 2\cdot44$	1819 ± 27	$1\cdot316 \pm 0\cdot007$
'Celtuce'	$0\cdot367 \pm 0\cdot0035$	$0\cdot208 \pm 0\cdot0029$	$65\cdot39 \pm 2\cdot12$	1764 ± 34	$1\cdot308 \pm 0\cdot002$
'Great Lakes 65'	$0\cdot374 \pm 0\cdot0059$	$0\cdot204 \pm 0\cdot0043$	$70\cdot50 \pm 1\cdot77$	1845 ± 31	$1\cdot292 \pm 0\cdot002$
'Oak Leaf'	$0\cdot371 \pm 0\cdot0081$	$0\cdot205 \pm 0\cdot0035$	$62\cdot24 \pm 3\cdot64$	1758 ± 41	$1\cdot306 \pm 0\cdot001$
'Saffier'	$0\cdot383 \pm 0\cdot0070$	$0\cdot219 \pm 0\cdot0052$	$75\cdot18 \pm 4\cdot53$	2089 ± 99	$1\cdot295 \pm 0\cdot004$
'Tetue de Nimes'	$0\cdot376 \pm 0\cdot0059$	$0\cdot207 \pm 0\cdot0037$	$62\cdot97 \pm 3\cdot28$	1814 ± 56	$1\cdot319 \pm 0\cdot004$
'Mataro Tres Ojos'	$0\cdot365 \pm 0\cdot0058$	$0\cdot204 \pm 0\cdot0051$	$64\cdot00 \pm 3\cdot05$	1669 ± 31	$1\cdot302 \pm 0\cdot004$
L. serriola	$0\cdot384 \pm 0\cdot0072$	$0\cdot207 \pm 0\cdot0044$	$63\cdot83 \pm 3\cdot56$	1867 ± 53	$1\cdot314 \pm 0\cdot007$
L. saligna	$0\cdot391 \pm 0\cdot0085$	$0\cdot223 \pm 0\cdot0077$	$58\cdot33 \pm 3\cdot35$	1727 ± 66	$1\cdot120 \pm 0\cdot004$
L. virosa	$0\cdot500 \pm 0\cdot0082$	$0\cdot187 \pm 0\cdot0054$	$81\cdot68 \pm 3\cdot78$	2131 ± 60	$1\cdot669 \pm 0\cdot007$

The differences in A_1 among the other accessions are not significant. *L. saligna* has the highest interchromosomal asymmetry index A_2, followed by *L. sativa* 'Saffier'. The remaining *L. sativa* accessions and *L. serriola* form a group with lower A_2 values than *L. saligna* and *L. sativa* 'Saffier', while only small differences in A_2 within this group

occur. *L. virosa* has the smallest A_2 of all of the accessions. Only the differences of *L. saligna* and *L. sativa* 'Saffier' versus *L. virosa* are significant. *L. virosa* has the largest total chromosome area, followed by 'Saffier', 'Great Lakes 65', 'Celtuce', 'Mataro Tres Ojos', 'Balady', *L. serriola*, 'Tetue de Nimes', 'Oak Leaf' and *L. saligna*, in order of decreasing area. The differences of *L. virosa* versus 'Celtuce', 'Mataro Tres Ojos', 'Balady', *L. serriola*, 'Tetue de Nimes', 'Oak Leaf' and *L. saligna* and that of *L. saligna* versus *L. sativa* 'Saffier' are significant.

   L. virosa and *L. sativa* 'Saffier' have the largest total chromosome length. The remaining accessions form a variable group and are in order of decreasing length: *L. serriola*, 'Great Lakes 65', 'Balady', 'Tetue de Nimes', 'Celtuce', 'Oak Leaf', *L. saligna* and 'Mataro Tres Ojos'. *L. virosa* is significantly different from all accessions within this group, apart from 'Saffier'. *L. sativa* 'Saffier' is significantly different from 'Balady', 'Tetue de Nimes', 'Celtuce', 'Oak Leaf', *L. saligna* and 'Mataro Tres Ojos'. None of the other accessions are significantly different from each other.

   L. virosa shows the highest and *L. saligna* the lowest relative DNA content of all accessions. The remaining accessions form a group with DNA contents intermediate between *L. saligna* and *L. virosa*. In order of decreasing DNA content these are: 'Tetue de Nimes', 'Balady', *L. serriola*, 'Celtuce', 'Oak Leaf', 'Mataro Tres Ojos', 'Saffier', 'Great Lakes 65'. *L. virosa* and *L. saligna* are significantly different from all of the other accessions. Within the remaining group of accessions only the differences between 'Tetue de Nimes' and 'Saffier', 'Tetue de Nimes' and 'Great Lakes 65' and between 'Balady' and 'Great Lakes 65' are significant.

   PCA results using A_1, A_2, total chromosome length, total chromosome area and relative DNA content are given in Fig. 3. The first axis (PC1), describing 54% of the variation, is composed of A_1, total chromosome area, total chromosome length and relative DNA content in about equal proportions, and by a smaller proportion of A_2. The second axis (PC2), accounting for 22% of the variation, is mainly determined by A_2 and smaller proportions of total chromosome length and relative DNA content. The third axis (PC3), which describes 16% of the variation, is mainly determined by A_1 and total chromosome area and smaller proportions of A_2 and relative DNA content.

   All *L. sativa* accessions form a single group, except for three aberrant 'Saffier' complements which are separated along the first and second principal component. The *L. serriola* complements are scattered among those of *L. sativa*. The *L. saligna* complements form a group that is only partially separated from the *L. sativa/serriola* group, along the first and second principal component. *L. virosa* occupies an isolated position mainly due to a separation along the first PC. *L. virosa* and *L. saligna* are the most dissimilar groups in the PCA.

DISCUSSION

Our data confirm that arm length differences between the subsequent chromosomes in the chromosome set of *Lactuca* are too small to avoid 'reversal of order' and 'arm reversal'. This makes unequivocal identification of the homologues on the basis of length and centromere position impossible and the karyotypes based on these identifications unreliable. However, the use of numerical parameters for the total cell complement avoids this identification problem.

   The karyotype of *L. virosa* as described by these numerical parameters differed from that of the other species in several respects. *L. virosa* had the largest intrachromosomal
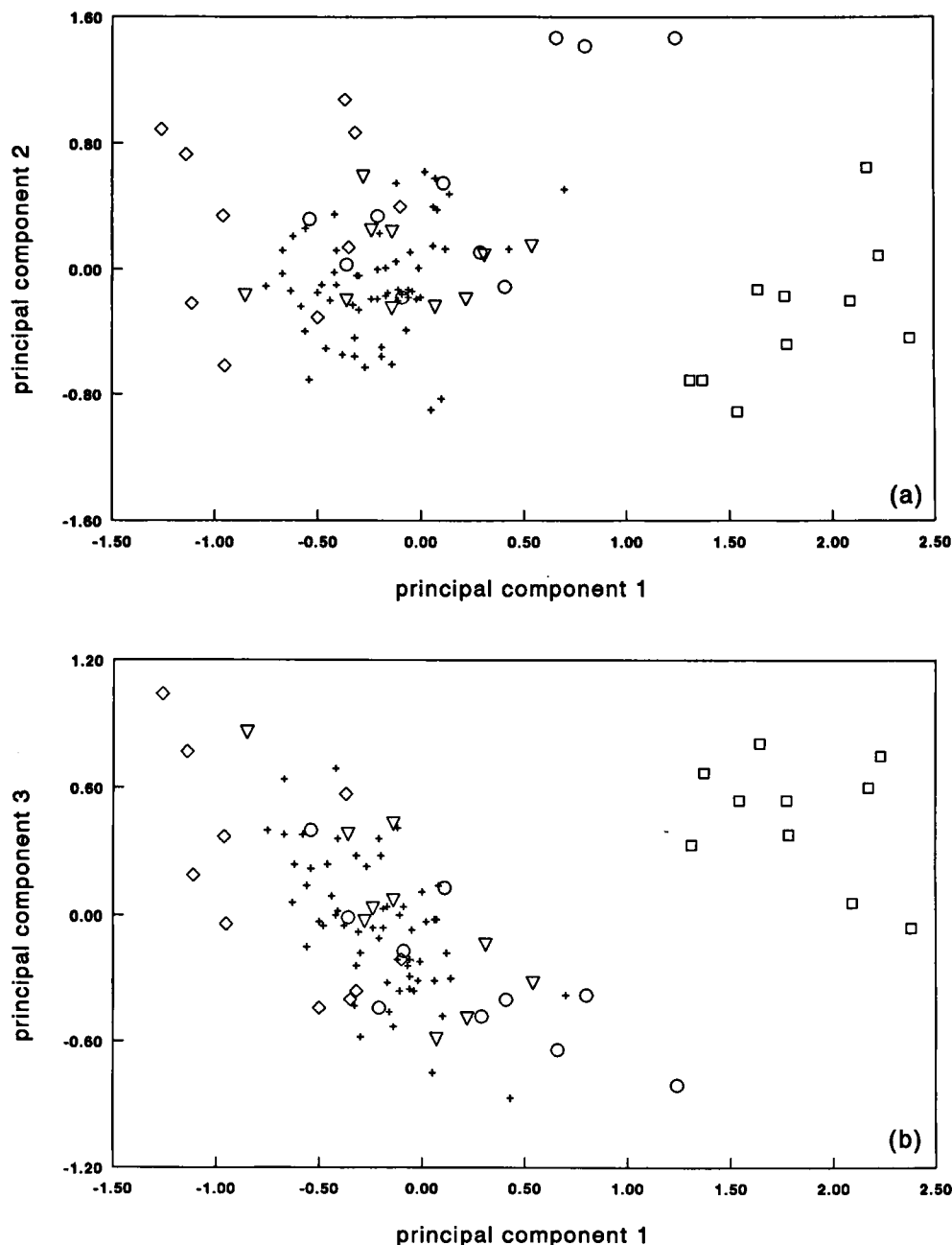
Fig. 3. Principal component analysis based on the parameters from Table 2. In both plots each of the 10 accessions is represented by 10 metaphases. ○, *L. sativa* 'Saffier'; +, *L. sativa*, remaining accessions; ▽, *L. serriola*; ◇, *L. saligna*; □, *L. virosa*.

asymmetry (A_1), the smallest interchromosomal asymmetry (A_2) and the largest genome in terms of total chromosome area and total chromosome length. *L. saligna* had a relatively asymmetric karyotype and a smaller genome. It had the second highest A_1, the

highest A_2, the smallest total chromosome area and the second smallest total chromosome length of all accessions. The karyotypes of *L. serriola* and all *L. sativa* accessions except *L. sativa* 'Saffier' were similar to each other and occupied an intermediate position between *L. virosa* and *L. saligna*, but closer to *L. saligna* than to *L. virosa*. For all accessions except *L. sativa* 'Saffier', the differences in relative DNA content showed a similar pattern.

Due to high mean values of A_1, A_2 and total chromosome length and area, the karyotype of *L. sativa* 'Saffier' differed from those of the other accessions in the *L. sativa/serriola* group (Table 2). The values of total chromosome length and area of the species are positively correlated with those of relative DNA content, suggesting a causality between the parameters. Since this is not the case for *L. sativa* 'Saffier', its large total chromosome length and area likely reflect a lower contraction degree at the time of fixation rather than a large amount of chromosome material. The PCA (Fig. 3) showed that this is caused by only three deviating complements. The results of *L. sativa* 'Saffier' are therefore not representative for a regular *L. sativa* karyotype and will be excluded from further discussion.

L. sativa and *L. serriola* cannot be discriminated by their karyotype or relative DNA content, as none of the parameters differed significantly between these species. *L. sativa/serriola* and *L. saligna* can only be discriminated on the basis of their relative DNA content, since they did not show any significant differences for the parameters describing the karyotype. *L. sativa/serriola* and *L. virosa* can be discriminated on the basis of A_1, total chromosome length and relative DNA content. *L. virosa* and *L. saligna* can be discriminated by all five parameters.

These results are in agreement with the conclusions of Lindqvist (1960), Chatterjee & Sharma (1969) and Haque & Godward (1985) that *L. sativa* and *L. serriola* have identical karyotypes. The karyotype of *L. virosa*, described by Lindqvist (1960) as containing more asymmetric chromosomes compared to that of *L. sativa/serriola* was confirmed by our results. The *L. saligna* karyogram established by Lindqvist (1960) shows chromosomes that are shorter and more unequal in length compared to those of *L. sativa/serriola*. Although none of the differences between *L. saligna* and *L. sativa/serriola* were found to be significant in our study, our data confirm Lindqvist's observations. Therefore his conclusion that the *L. saligna* karyogram is only slightly different from that of *L. sativa/serriola* is supported. In accordance with Lindqvist's observations two pairs of satellites were observed in *L. virosa* and four pairs in *L. sativa*, *L. serriola* and *L. saligna*. The variation in mean number of visible satellites among the accessions can be explained by differences in the state of despiralization of the secondary constrictions in part of the nucleolar organizing chromosomes. If the constrictions are completely condensed, the microsatellites remain tightly attached to the chromosome and are therefore invisible. The extent of despiralization of the secondary constriction reflects metabolic activity of that region rather than polymorphisms for the satellite and so makes the number of visible microsatellites inappropriate for using as a taxonomic parameter. Therefore these differences will not be given any further consideration.

In his discussion on karyotype symmetry in relation to phylogeny and evolutionary processes, Stebbins (1971) assumed a predominant evolutionary trend towards increasing asymmetry in the karyotype. Although opposite trends occur in specific genera (Stebbins 1971; Jones 1978), the trends towards increasing asymmetry are particularly obvious within the Compositae, tribe Cichorieae (including *Lactuca*) (Babcock 1947; Stebbins *et al.* 1953).

Since all four *Lactuca* species in our study have 18 chromosomes, differences in their karyotypes can be ascribed to processes which do not influence the chromosome number, such as rearrangements within the chromosome arms, pericentric inversions and unequal translocations. Lindqvist (1960) found no multivalents in interspecific hybrids within the subsect. *Lactuca*. Therefore he concluded that the differences in chromosome structure among the species of subsect. *Lactuca* originated in pericentric inversions rather than in translocations. Since this is a process driving a primary trend towards increasing asymmetry, the most asymmetric of the karyotypes in our study, namely that of *L. virosa* and *L. saligna*, can be considered the most derived. Because these are also the most dissimilar karyotypes, they apparently evolved in different directions. Alternatively, gradual deletions and/or duplications of repetitive sequences, and so of some heterochromatin classes may contribute to the shift of centromeres. The differences in banding patterns between *L. sativa/serriola*, *L. saligna* and *L. virosa* as shown by Koopman *et al.* (1993) are in favour of this assumption.

Using the karyotype parameters and relative DNA contents to characterize the species it can be concluded that:

1. *L. sativa* and *L. serriola* are very closely related or even conspecific, *L. saligna* is a dissimilar but not too distinct species and *L. virosa* is clearly separate from the other three. This is in accordance with data on chromosome banding patterns (Koopman *et al.* 1993) and crossability (De Vries 1989). The status of *L. virosa* as a separate species is confirmed by numerical morphological analyses (Frietema de Vries *et al.* 1994; De Vries & Van Raamsdonk 1994) as is that of *L. saligna* (De Vries & Van Raamsdonk 1994). The clusters of *L. sativa* and *L. serriola* showed a slight overlap in these analyses. Frietema de Vries *et al.* (1994) considereed this overlap large enough to lump the species, while De Vries & Van Raamsdonk (1994) considered it small enough to maintain them. Our results do not support the distinction of *L. sativa* and *L. serriola* as separate species.

2. *L. saligna* and *L. virosa* are the most dissimilar of the four species examined, while the karyotype morphology and relative DNA content of *L. sativa* and *L. serriola* are intermediate between that of *L. saligna* and *L. virosa*. Crossability data (De Vries 1989) support this position of the species relative to each other. Morphological data only support the intermediate position of *L. serriola* relative to *L. saligna* and *L. virosa*. *L. sativa* occupied a different position in morphological analyses, partly due to the presence of characters caused by domestication (De Vries & Van Raamsdonk 1994). Apparently, the domestication process is not reflected in the karyotype.

Results on DNA and enzyme analyses are not in accordance with the data on karyotype, relative DNA content, banding patterns, morphology and crossability. RFLP analysis of nuclear DNA shows a closer similarity of *L. saligna* and *L. virosa* to each other than to *L. sativa* and *L. serriola* (Kesseli *et al.* 1991). Analysis of mitochondrial RFLPs (Vermeulen *et al.* 1994) makes clear that *L. sativa* and *L. serriola* share more mtDNA fragments with *L. virosa* than with each other, while all three species shared the least fragments with *L. saligna*. Results on isozyme analysis (Kesseli & Michelmore 1986) showed clusters containing *L. sativa*, *L. saligna*, *L. virosa*, *L. serriola*, *L. virosa* and *L. serriola*, in order of increasing Nei's genetic distance relative to *L. sativa*. Considering the fact that the *L. saligna* and *L. virosa* accessions showing the smallest genetic distance to *L. sativa* were not identified with certainty, it must be concluded that the isozyme results give no unequivocal picture of the species relationships. De Vries (1996) showed that SDS-electrophoresis patterns of achene proteins from *L. sativa* and *L. serriola* were similar, while the patterns of *L. saligna* and *L. virosa*

differed from that of *L. sativa/serriola* and from each other. The *L. saligna* pattern was the most dissimilar from that of *L. sativa/serriola*. As becomes clear from the contradictory results mentioned above, further research will be needed to obtain a more obvious view of the relationships among *L. sativa*, *L. serriola*, *L. saligna* and *L. virosa*.

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