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Research Article

Contribution of leaf morphometrics in the study of genetic entries in *Salix* L.

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Abstract:
The use of leaf morphometrics in *Salix* was investigated at three genetic entry levels: species, family and clonal (progeny genotype). Data for seven leaf parameters, were collected from four full-sib *Salix eriocephala* and three *S. exigua* families. Principal component analysis, multivariate analysis of variance and discriminant analysis showed that most of the variation was resolved in low multidimensional space. Leaf and stipule shape parameters emerged as most important variables. Separation at the family level was partially achieved in a tri-dimensional ordination of the major axes in both principal and discriminant space. Interspecific distances were 21 times greater than intraspecific ones on the average, indicating very good separation of *S. eriocephala* from the taxonomically troublesome *S. exigua*. A maternal effect at the family level was detected by all analyses, while discrimination at the clonal level was not possible.

Key words:
Willows, leaf morphology, full-sib family, identification

Introduction

The willows (*Salix* L.; Salicaceae), consist of over 500 species (Argus, 1986) and are well known for their wide distribution, variability and vitality (Zsuffa, 1990). Willows were recognized as an alternative source of energy and willow culture faces a growing interest in many countries especially for industrial short rotation biomass plantations (Zsuffa, 1990). Willows have not been exposed so far to extensive genetic studies, presenting an almost intact genetic reserve. Discrimination at the species, family and clonal level in perennial woody angiosperms has been traditionally exercised through the use of morphological and phenological descriptors. Within the Salicaceae they have been used successfully in poplars, providing a means for clonal description that does not depend on infrastructure and financial constrains (UPOV, 1981; Roller, 1984).This paper reports the use of leaf morphometrics concerning in two willow species, *S. eriocephala* Muhl. and *S. exigua* Nutt., important for biomass production (Zsuffa, 1990). Because of their availability and the wide range of morphological variation that leaves exhibit, leaf form has been a rich source of identification and discrimination data for as long as plants have been classified (Theophrastus, 1990). Leaf differences are due to genetic control of morphogenesis, contrasting environmental conditions during development, normal developmental pattern (leaf heteroblasty), shoot types within and between plants, degree of heterozygosity, etc. (Paxman, 1956). Leaf morphometrics have been used in several applications besides taxonomy and systematics, such as in studies of population or provenance differentiation (Dickinson, 1986, Aravanopoulos, 2005) and hybridization (Neophytou, *et al*., 2007), etc. The patterns of divergence shown by leaf characteristics proved to be taxonomically most important in *Populus* (Eckenwalder 1977, Anonymous 1979) and have been used in population variation studies of *Populus deltoides* (Rajora *et al*., 1991). In willows pertinent information is very scarce; there are only two studies that have used leaf morphometrics to study *Salix*

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evolution from extant leaves and fossil leaf imprints (Thiebaut, 2000 and 2002). This investigation is concentrated at three genetic levels: species, family and clone.

Material and Methods

Two species *S. eriocephala* (subgenus *Vetrix*, section *Cordatae*, 2n=38) and *S. exigua* (subgenus *Salix*, section *Longifoliae*, 2n-38) and seven full-sib families (four of the former species and three of the latter) were used. Families followed a complete 2 x 2 factorial mating design in *S. eriocephala* and a corresponding incomplete mating design in *S. exigua* (Table 1). Parental clones originated from unrelated populations of South-Eastern Ontario (Mosseler 1989), while all plant material has been growing in a family trial under the same conditions and treatments (Mosseler 1989, Zsuffa 1990). Twelve plants per family and ten leaves per plant were used. The parameters measured were: LL: lamina length (cm), LW: lamina width (cm), PL: petiole length (cm), BW: distance from leaf base to the leaf maximum width (cm), TC: number of teeth per centimetre, SL: stipule length (cm), SW: stipule width (cm). Summary descriptive statistics at the family level were computed. Data normality was checked by means of the coefficient of variation (CV) and the Shapiro-Wilk statistic (W). Data transformations were performed when normality criteria were not met. Then, the product moment correlation coefficient matrix of the variables provided a criterion for the elimination of the highly correlated variables.Multivariate analysis was employed in order to examine the simultaneous contribution of all leaf parameters in discriminating between species and families within species. Firstly, principal component analysis (PCA) was performed to depict group ordination in principal space. In the fields of morphometrics and numerical taxonomy the use of PCA is widespread, since it fits for the analysis of multiple variables per individual data and it results in sensible biological explanation of the results (Pimentel 1979, Dunn and Everitt 1982). In component analysis the original variables are transformed to variables that have zero intercorrelations. The transformation rotates the original axes but maintains the original relationships among data points. The new axes define independent patterns of variation infrequently recognized as size and shape variables that should typify the population sampled (Pimentel 1979). PCA was conducted on the correlation matrix since this PCA separates size from shape more effectively than the analysis of the covariance matrix (Somers 1986).

The genetic entry level group of full-sib families was also subjected to a multiple discriminant analysis (MDA; Marriot 1974, Pimentel 1979), in order to investigate if the resulting classification was in agreement with the original leaf data. Initially data were subjected to a multivariate analysis of Variance (MANOVA) to check the significance of among group differences (test of H_0: equality of group centroids). When family differences were revealed, MDA was employed to: (a) explain differences between groups in terms of a linear compound of p explanatory variables which maximize separation, and (b) classify individuals with minimum probability of misclassification. MDA provides insight into the relative importance of variables which separate categories, the main purpose of the technique being to identify misclassified individuals (Pimentel 1979). The percentage of return (Pimentel 1979) to original groups after ordination in discriminant space is a measure of the true relationship of leaf variables to the family groups. To examine the separation between families the Mahalanobis generalized distance function (D^2) was chosen. The SAS software (SAS 2000) was used in all analyses.

Results and Discussion

The mean, standard deviation and coefficient of variation for the morphological variables studied are presented in Table 2. Due to a general lack of normality of row data, several transformations were attempted, the logarithmic ($\log_{10}$) being better. This transformation provided a further advantage to the analysis, as linearity and multivariate normality are more closely approximated by logarithms than by the original variables, while logarithms tend to provide components that are independent of scales of measurement and order of magnitude of variances (Pimentel 1979, Dickinson 1986, Sommers 1986). According to the product moment correlation coefficients matrix, lamina width was found to be highly correlated with lamina length (results not presented). Hence the fraction LL/LW, defined as lamina shape (LS) was used instead. This ratio which is an independent shape variable has been used extensively in leaf morphometrics (Dickinson 1986, Dickinson et al. 1987, Aravanopoulos 2005).Six variables (LS, PL, BW, TC, SL, SW) were used in PCA and were transformed into six different vectors of variation. The eigenvalues, percent of trace and cumulative percent of the principal components are presented in Table 3. The major proportion of variation (87.64%) is accounted for by the first two principal axes. All components were bipolar (Table 4) and they could be interpreted as shape components (Pimentel 1979). The deflection point of a plot of eigenvalues (not shown) indicated that the examination of two components is adequate for data interpretation. The first component separated *S. eriocephala* from *S. exigua* (Fig. 1). This component is characterized by the high loadings of LS, PL, SL and SW (Table 4). Additionally there was some separation between the *S. eriocephala* families, as families E16xE263 and E16xE292 were separated from families E269xE263 and E269xE292. This separation was more distinguishable when the second component (characterized by the high loading of BW) was considered too. On the other hand the individuals of the *S. exigua* families (I61xI66, I62xI66, I61xI293) formed a single cluster. Within family separation is more distinct in the third vector which is significantly influenced by the high loading of LS (Fig. 1).

MANOVA rejected the null hypothesis of equality of group centroids (Wilks' Lambda W=0.004, $p<0.001$), indicating the validity of the MDA approach. The first axis in discriminant space accounted for 91.6% of the total variation while the first two axes accounted for almost all the variation (98.1%, Table 5). The first axis was characterized by the high loadings of SW while in the second LS presented the highest eigenvector (Table 5). The generalized squared distances between families are presented in Table 6. Mahalanobis distances were large enough to separate at least different groups of families within a species. Families of *S. eriocephala* were better separated than those of *S. exigua* and their D^2 values were greater, however families E16xE263 and E16xE292 are closer to each other and the same is observed for families E269xE263 and E269xE292, as well as for families I61xI66, I62xI66 and I61xI293 (Table 6). Results fully support the PCA outcome. The two different species were very well separated and interspecific distances were 21 times greater than intraspecific ones on the average (Table 6). The two species belong to different subgenera, however *S. exigua* has been considered as being equally divergent from both willow subgenera *Salix* and *Vetrix* (Chong et al 1995). The leaf morphometric results indicate a very good separation of *S. eriocephala* from the taxonomically troublesome *S. exigua*. In Table 7, the classification and redistribution summaries are presented. In conformity with previous results, all reclassifications were within the groups defined by PCA and Mahalanobis distance.

Most of the leaf quantitative variation has been resolved in the first two components both in principal and in discriminant space. The two willow species separated very well on the first axis. Lamina shape and stipule parameters characterize the first principal component, while stipule width is highly loaded on the first discriminant component. Therefore leaf and stipule shape appear to discriminate well at the species level. At the genetic level of family, separation is achieved on the second (high loads of BW) and third components (LS highly loaded). However, the genetic entry of the clonal level could not be separated.The ordination of families into principal and discriminant space and the Mahalanobis D^2 values indicated the formation of particular family groups. As a property of the factorial mating design, families share either the maternal or the paternal parent (Table 1). Results showed that a family is closer to the one with which it shares the same maternal parent than to the family with which the same paternal parent is shared (Fig 2.). For example, family E16xE263 is closer to E16xE292 (common maternal parent E16), than to E269xE263 (common paternal parent E263). Moreover this observation is further supported by the MDA reclassification results (Table 7). Plants from a family which were characterized as misclassified have been reclassified only to the family in which the maternal parent is common in *S. eriocephala*, or primarily to this family in *S. exigua*. Note that the interspecific family I61xE263 is close to the *S. exigua* families (having a *S. exigua* maternal parent). It seems that there is a maternal effect on the offspring of these *Salix* controlled crosses. This conclusion supported by the inclusive multivariate techniques used, is in congruence to the work of Mosseler (1989) who suspected this kind of influence from studies in *Salix* phenology. Maternal effects have not been studied extensively in Salix, but they have been implied in a study of *S. sericea* x *eriocephala* progeny (Orians et al. 2000). Maternal effects can have a substantial influence on offspring phenotype and strongly affect the execution of breeding programs (Roach and Wulff 1987, Galloway and Etterson 2007). The distinction of species at the genetic entry level based on leaf morphometric analysis was very good. At the family level, separation was partial. Families were separated according to their maternal parent, but not further. Evidently, there was no distinction at the clonal (progeny genotype) level. In conclusion, *Salix* leaf morphometrics were adequate in discriminating at the species level and partially at the family level. The contribution of leaf morphometrics in studying different genetic entry levels in Salix is useful, albeit limited. Especially clonal identification would require the use of completely penetrant genetic markers.

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Table 1. Mating design of *S. eriocephala* and *S. exigua* full-sib families used in this study (E: *S. eriocephala* parental clones, I: *S. exigua* parental clones)

	E263	E292	I66	I293
E16	X	X		
E269	X	X		
I61			X	X
I62			X	

Table 2. Descriptive statistics of *Salix* morphological variables at the family genetic entry level

Family	Statistic	LL	LW	PL	BW	TC	SL	SW
E16xE263	Mean	11.75	3.06	1.13	5.54	4.43	1.53	0.68
	SD	1.11	0.36	0.21	0.65	0.92	0.31	0.10
	CV	9.43	11.70	18.91	11.78	20.83	20.13	15.33
E16xE292	Mean	11.05	2.30	1.12	4.70	5.49	1.42	0.60
	SD	1.19	0.43	0.29	0.91	1.07	0.37	0.18
	CV	10.77	15.25	25.66	19.47	19.43	26.11	29.14
E269xE263	Mean	11.99	2.63	1.18	4.61	6.31	1.32	0.53
	SD	1.21	0.35	0.23	0.84	1.18	0.24	0.11
	CV	10.09	13.37	20.53	18.25	18.85	18.59	21.19
E269xE292	Mean	11.56	2.63	1.08	5.01	6.85	1.32	0.56
	SD	1.12	0.32	0.22	0.74	1.06	0.24	0.12
	CV	9.73	12.19	20.60	14.85	15.44	17.99	21.22
I61xI293	Mean	10.11	0.65	0.32	0.46	2.20	0.08	0.05
	SD	1.53	0.18	0.11	0.27	0.78	0.18	0.14
	CV	15.56	28.06	33.55	58.15	35.41	213.54	256.54
I61xI66	Mean	12.15	0.61	0.29	0.36	2.29	0.20	0.16
	SD	2.15	0.29	0.11	0.28	0.90	0.25	0.22
	CV	17.74	47.63	37.87	76.91	39.35	122.80	137.14
I62xI66	Mean	11.27	0.73	0.39	0.47	2.02	0.01	0.003
	SD	1.26	0.28	0.20	0.30	0.70	0.08	0.02
	CV	11.17	38.71	30.53	64.72	34.45	583.39	571.49

LL: lamina length; LW: lamina width; PL: petiole length; BW: distance from leaf base to the leaf maximum width; TC: number of teeth per centimetre; SL: stipule length; SW: stipule width

Table 3. Principal components analysis at the *Salix* family genetic entry level: eigenvalues of the correlation matrix

Principal Axis	1	2	3	4	5	6
Eigenvalue	4.331	0.927	0.393	0.185	0.144	0.020
Percent of Trace	72.189	15.447	6.547	3.082	2.408	0.326
Cumulative percent	72.189	87.637	94.184	97.266	99.674	100.000

Table 4. Principal components analysis at the *Salix* family genetic entry level: eigenvectors

Principal Axes	1	2	3	4	5	6
LS	-0.432	-0.006	0.542	0.266	0.648	0.165
PL	0.447	0.032	-0.286	-0.649	0.608	-0.075
BW	0.174	0.959	0.190	0.092	-0.071	-0.002
TC	0.393	-0.247	0.803	-0.099	-0.353	-0.071
SL	0.455	-0.103	-0.106	0.630	0.271	-0.548
SW	0.470	-0.084	-0.113	0.302	0.077	0.813

LS: Lamina shape; LW: lamina width; PL: petiole length; BW: distance from leaf base to the leaf maximum width; TC: number of teeth per centimetre; SL: stipule length; SW: stipule width

Table 5. MANOVA at the *Salix* family genetic entry level: eigevalues and associated eigenvectors for the first three axes

Axis	Eigenvalue	Percent of Trace	Cumulative Percent	Eigenvectors		
				LS	PL	BW
				TC	SL	SW
1	39.760	91.60	91.60	0.139	-0.031	0.045
				-0.012	-0.387	0.092
2	3.087	7.110	98.71	0.166	0.075	-0.032
				0.023	0.153	-0.006
3	0.310	0.710	99.42	0.013	-0.185	0.108
				-0.006	-0.032	0.158

LS: Lamina shape; LW: lamina width; PL: petiole length; BW: distance from leaf base to the leaf maximum width; TC: number of teeth per centimetre; SL: stipule length; SW: stipule width

Table 6. Pairwise Generalized Squared Distances at the *Salix* family genetic entry level; D^2 values obtained from Mahalanobis distance function analysis for leaf morphological characteristics.(*)

From Family	I	II	III	IV	V	VI	VII
I	0						
II	2.653	0					
III	31.349	26.368	0				
IV	31.887	26.973	0.760	0			
V	205.852	201.620	126.886	137.009	0		
VI	210.722	206.048	128.389	139.117	1.850	0	
VII	189.297	185.253	121.333	132.012	5.626	3.148	0

(*) I: E16xE263 III: E269xE263 V: I61xI66 VII: I62xI66
II: E16xE292 IV: E269xE292 VI: I61xI293

Table 7. MDA at the *Salix* family genetic entry level: classification summary for calibration data, resubstitution summary using the linear discriminant function, number of observations and percent classified into the family level.(*)

From Family	I	II	III	IV	V	VI	VII
I	95	25	0	0	0	0	0
	79.17	20.83	0.00	0.00	0.00	0.00	0.00
II	32	88	0	0	0	0	0
	26.67	73.33	0.00	0.00	0.00	0.00	0.00
III	0	0	79	39	0	0	1
	0.00	0.00	66.39	32.77	0.00	0.00	0.84
IV	0	0	45	75	0	0	0
	0.00	0.00	37.50	62.50	0.00	0.00	0.00
V	0	0	0	0	61	25	34
	0.00	0.00	0.00	0.00	50.83	20.83	28.33
VI	0	0	0	0	29	65	26
	0.00	0.00	0.00	0.00	24.17	54.17	21.67
VII	0	0	0	0	4	10	86
	0.00	0.00	0.00	0.00	4.00	10.00	86.00

(*) I: E16xE263 III: E269xE263 V: I61xI66 VII: I62xI66
II: E16xE292 IV: E269xE292 VI: I61xI293

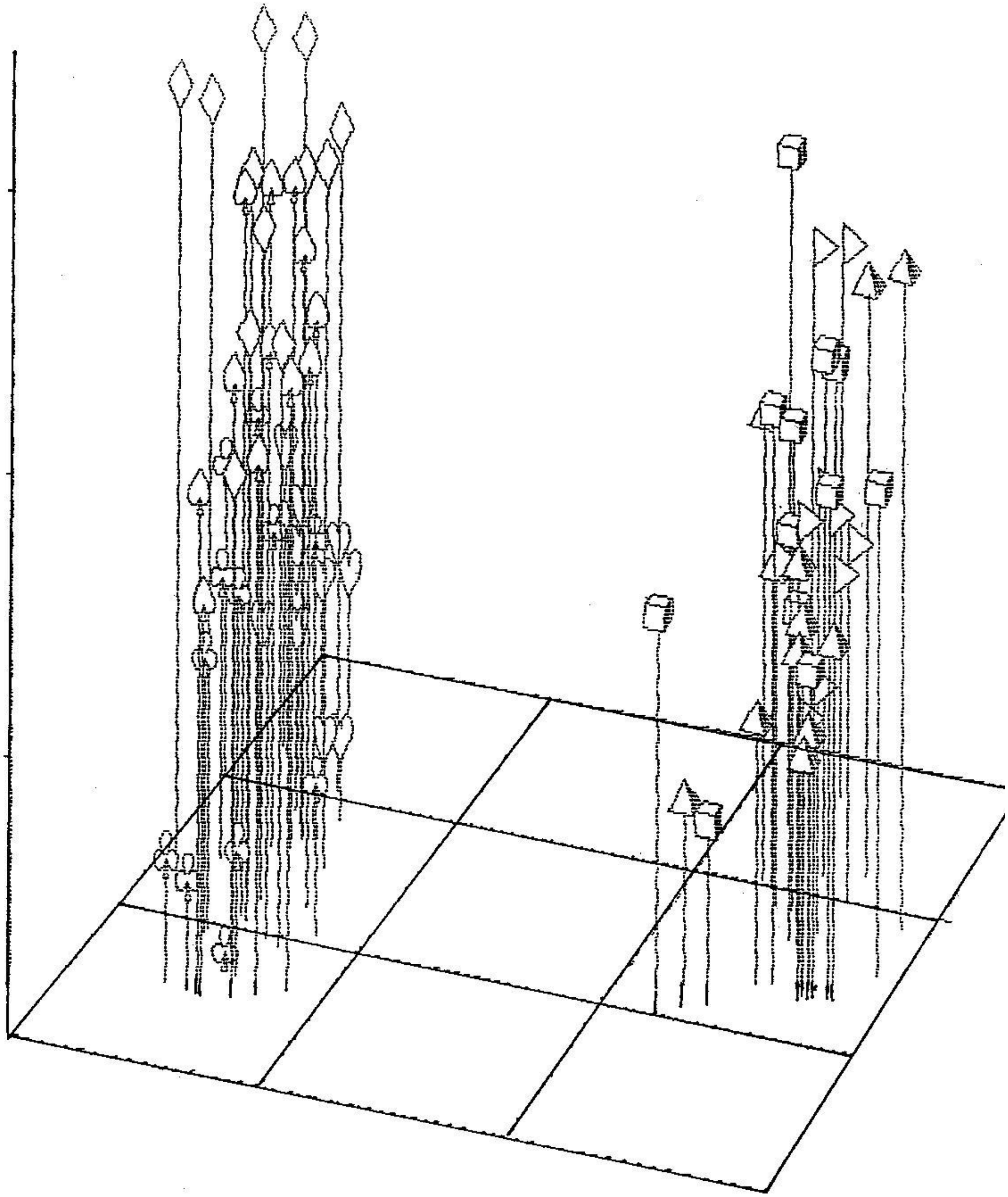

Figure 1. PCA plot of the first three principal components (DIAMOND: E16xE263, HEART: E269xE263, SPADE: E16xE292, CLUB: E269xE292, CUBE: I61xI66, PYRAMID: I62xI66, FLAG: I61xI293).

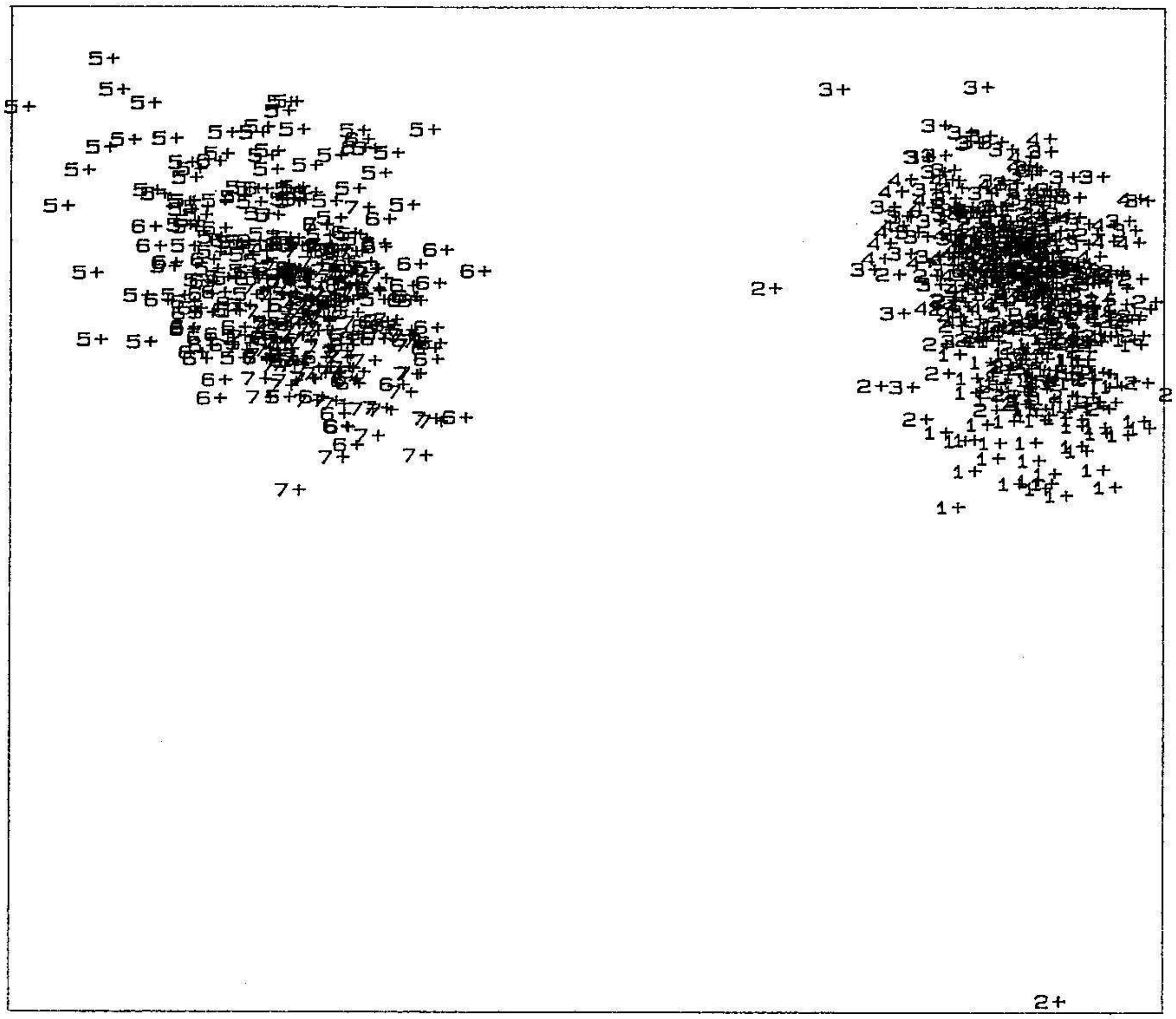

Figure 2. MDA: ordination of the first two axes; original classification (1: E16xE263, 2: E269xE263, 3: E16xE292, 4: E269xE292, 5: I61xI66, 6: I62xI66, 7: I61xI293).