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Relationships among *Agropyron, Dasypyrum* and *Lophopyrum* (Triticeae: Poaceae) viewed from isoenzyme variation of esterase, peroxidase and acid phosphatase

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Isoenzyme variation of esterase, peroxidase and acid phosphatase was examined in individual plants from natural populations of *A. cristatum, D. villosum* and *L. elongatum.* Four indices of phenotypic similarity (SI, S, D, I_h) were calculated in an attempt to assess relationships among the taxa studied. All indices have their lowest estimates in the comparison between *L. elongatum* and *D. villosum*. This is an indication that both species are most distantly related as judged by the enzymes surveyed. Although slightly higher, the values of SI, S and I_h show that *D. villosum* and *A. cristatum* are also positioned remotely. *Dasypyrum. villosum* possessed the highest number of diagnostic isoforms, which is an additional indicator of its distinctness within the studied group. The species *A. cristatum* and *L. elongatum* demonstrate a higher level of affinity as estimated by all indices used. The proposed relationships among *Agropyron, Dasypyrum* and *Lophopyrum* are compared with conclusions derived from morphological and molecular data available.

Key words: isoenzymes, esterase, peroxidase, acid phosphatase, *Agropyron, Dasypyrum, Lophopyrum*

Introduction

The tribe Triticeae contains wheat (*Triticum* L.), barley (*Hordeum* L.) and rye (*Secale* L.) which are economically the most important cereals in the temperate zone of the globe. Additionally, the tribe includes a number of forage grasses. Many wild species are a rich source of genes for desirable traits such as disease resistance, salt and drought tolerance, which are of interest to breeders of cultivated grasses. Due to widespread hybridization and polyploidy within the tribe Triticeae, its evolutionary history is complicated and there is still little agreement on the phylogeny and taxonomy of the tribe. For its importance, the group has been at the focus of numerous phylogenetic and evolutionary studies considering morphological (BAUM 1983, BAUM et al. 1987, FREDERIKSEN and SEBERG 1992, SEBERG and

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FREDERIKSEN 2001), cytogenetical (HSIAO et al. 1986, WANG 1989) and molecular characteristics (DVORAK and APPELS 1982, JARVIE and BARKWORTH 1990, BAUM and APPELS 1992).

In some cases molecular data pose new questions, rather than resolving the relationships among particular genera within Triticeae. Based on similarity of morphology, some authors consider *Dasypyrum* a relative of *Triticum, Aegilops* and *Secale*. Crosses of *Dasypyrum* with different species of *Triticum, Aegilops* and *Secale* have been attempted (BLANCO et al. 1983, JAN et al. 1986, LUCAS and JAHIER 1988). Supposed spontaneous hybrids between *Dasypyrum* and *Triticum* have also been reported (STEFANI et al. 1987). In a cladistic analysis of Triticeae based on morphology (KELLOGG 1989), *Dasypyrum* was placed near *Agropyron* and *Crithodium (Triticum)*. Two molecular data sets of chloroplast DNA (cpDNA), one based on restriction site variation (MASON-GAMER and KELLOGG 1996a) and the other on sequences encoding the -subunit of RNA polymerase (PETERSEN and SEBERG 1997), placed *Lophopyrum (Thinopyrum)* and *Dasypyrum* together on cpDNA cladograms. More recently, sequencing of the nuclear starch synthase gene has also revealed a close affinity between *Lophopyrum* and *Dasypyrum* (MASON-GAMER and KELLOGG 2000). The nuclear DNA data (HSIAO et al. 1995, KELLOGG and APPELS 1995) are incongruent with the cpDNA data, as these studies suggest different affinities of *Lophopyrum* and *Dasypyrum* within Triticeae.

Evidently, there are considerable discrepancies among different studies treating the relationships among *Agropyron, Dasypyrum* and *Lophopyrum.* To address the problem further, *Agropyron cristatum* (L.) Gaertner (P genome), the V genome species *Dasypyrum villosum* (L.) P. Candargy (*Haynaldia villosa* (L.) Shur, cfr. HUMPHRIES 1978) and the E genome species *Lophopyrum elongatum* (Host) A. Löve (synonymous names *Thinopyrum elongatum* (Host) D. R. Dewey, *Elytrigia elongata* (Host) Nevski, *Agropyron elongatum* (Host) Beauv.) were examined for isoenzyme variation of esterase, peroxidase and acid phosphatase. The proposed relationships were compared with conclusions derived from the morphological and molecular data available.

Material and Methods

The enzymes esterase (EST, EC 3.1.1.1.), peroxidase (PER, EC 1.11.1.7.) and acid phosphatase (ACP, EC 3.1.3.2.) were analysed in individual plants from three populations of *A. cristatum,* four populations of *D. villosum* and three populations of *L. elongatum* (Tab. 1). Vouchers are deposited at the herbarium of the Institute of Botany (SOM).

Leaves were ground in 0.01 M Tris, 0.08 M. glycine, 0.005 M cysteine, Dowex 1 8 (0.4 g / g fresh tissue), 20% sucrose, pH 8.3. Homogenates were centrifuged at 10000 rpm for 10 minutes. The supernatant was used as a source of enzymes. Anodal isoforms of EST and ACP were resolved on 7.5% polyacrylamide slabs (3% stacking gel) according to DAVIS (1964). Cathodal isoforms of EST and PER were analysed on 7.5% separating and 3% stacking gel by the electrophoretic system of REISFELD et al. (1962). The length of the separating gel was 6 cm (5 cm for ACP) and stacking gels were 1.5 cm long. Electrophoresis of cathodal EST and PER was carried out until the indicator dye, pyronin G, reached the gel end (1 front). The duration of anodal electrophoresis was 1.25 fronts of indicator bromphenol blue for EST and 1.5 fronts for ACP. Staining protocols were performed as described earlier (ANGELOV 2000). Each isoform was assigned a number which reflects its gel migration in mm from the origin (PEREZ DE LA VEGA and ALLARD 1984).

Tab. 1. Species and populations studied. N – number of invidual plants/population examined

Species	Localities in Bulgaria	N	Voucher
Agropyron cristatum	Golo Burdo Mt. around Stoudena village	32	Co-330
	Thracian Lowland, Besapara hills, Ognyanovo	29	Co-329
	Thracian Lowland, Besapara hills, around village of Sinitevo	30	Co-386
Dasypyrum villosum	Thracian Lowland, Besapara hills, near Ognyanovo village	25	Co-624
	Sredna gora Mt., near Chavdar village	27	Co-626
	Strouma valley region, Marikostinovo village	33	Co-600
	Sredna gora Mt., near Chelopech village	24	Co-627
Lophopyrum elongatum	Southern Black Sea coast, around the town of Pomorie	22	Co-604
	Strouma valley region, the village of Marikostinovo	32	Co-605
	Southern Black Sea coast, around the town of Nesebar	26	Co-400

Enzyme systems EST, PER and ACP display considerable variation in total number of isoenzymes expressed, number of subunits per enzyme and the presence of »null« alleles (TANKSLEY and RICK 1980, SCHMIDT and WEHLING 1983, BREWBAKER et al. 1985, CHERISEY et al. 1985). These circumstances make genetic interpretation without formal genetic analysis difficult. For these reasons, instead of allelic frequencies, the percentage of occurrence of each isoform (electrophoretic band) in a given population was determined. Data about individual populations were combined for each species and mean frequencies were calculated.

Based on the presence/absence and frequency data, mean values of four measures of phenotypic similarity were calculated:

1) Similarity index (SI) of Jaccard (cfr. CHUNG et al. 1991)

$$SI = \frac{M}{M \quad N}$$

where M is the number of isoforms common for both taxa compared and N is the sum of species specific isoforms.

2) Coefficient of similarity (S) of Socal and Sneath (cfr. KALINOWSKI et al. 1979)

$$S = \frac{a \quad d}{a \quad b \quad c \quad d}$$

where a is the number of isoforms common for both taxa, b and c – the number of isoforms specific for each taxa compared, d – the number of isoforms absent from both taxa compared.

3) HEDRICK's (1971) measure of phenotypic identity:

$$I_h = 2I_{xy} / I_x + I_y$$

where $I_{xy} = \sum_{j\ 1}^{n} P_{jx} P_{jy}$; $I_x = \sum_{j\ 1}^{n} P^2_{jx}$ and $I_y = \sum_{j\ 1}^{n} P^2_{jy}$,

P_{jx} and P_{jy} are the frequencies of jth isoform in species x and y, and n is the number of enzyme isoforms.

4) Biochemical distance (D) of Socal and Sneath (cfr. STUESSY 1990):

$$D = \frac{1}{N} \sum_{i\ 1}^{N} \left(x_{ij} \quad x_{ik} \right)^{2\ \frac{1}{2}}$$

where N is the number of enzyme isoforms, x_{ij} and x_{ik} – the frequency of ith isoform in taxa j and k.

Results

In total, fifteen isoforms of anodal EST were electrophoretically detected (Tab. 2). Four isoforms (18, 21, 33, 41) were shared by all species examined. Isoforms 39 and 43 were diagnostic for *A. cristatum,* whereas *D. villosum* and *L. elongatum* possessed two (24, 38) and one (30) specific isoforms, respectively. Pair-wise comparisons among the species resulted in values of SI and S between 0.46 and 0.62.

Tab. 2. Mean isoform frequencies of anodal esterase in the studied populations of *Agropyron cristatum, Dasypyrum villosum* and *Lophopyrum elongatum.* Each isoform was assigned a number which reflects its gel migration (in mm) from the origin.

Species	Isoforms														
	16	18	21	24	26	28	30	33	35	37	38	39	41	43	45
A. cristatum	0.48	0.70	1.00	0.00	0.00	0.00	0.00	0.37	1.00	1.00	0.00	1.00	1.00	1.00	1.00
D. villosum	0.16	1.00	0.23	1.00	0.56	0.85	0.00	0.27	0.00	0.00	0.43	0.00	1.00	0.00	1.00
L. elongatum	0.00	0.57	1.00	0.00	0.15	0.45	0.75	0.12	1.00	0.12	0.00	0.00	1.00	0.00	0.00

Isoenzyme structure of the species in respect to cathodal EST is presented in Tab. 3. Of five isoforms detected, one isoform (30) was shared by all species. Isoforms 18 and 42 occurred in *D. villosum* only. Isoform 34 was diagnostic for *L. elongatum.* Similarity indices SI and S ranged from 0.17 (*L. elongatum* vs. *D. villosum*) to 0.66 in comparison between the latter species with *A. cristatum*.

Tab. 3. Mean isoform frequencies of cathodal esterase in the studied populations of *Agropyron cristatum, Dasypyrum villosum* and *Lophopyrum elongatum.* Each isoform was assigned a number which reflects its gel migration (in mm) from the origin.

Species	Isoforms					
	18	25	30	34	40	42
A. cristatum	0.00	0.48	0.69	0.00	0.74	0.00
D. villosum	0.65	0.42	0.92	0.00	0.00	0.89
L. elongatum	0.00	0.00	0.63	0.12	0.12	0.00

Eleven isoforms of cathodal PER were resolved (Tab. 4). Isoforms 10, 14, 36 and 40 were found in all species studied. Two isoforms (5, 28) were characteristic of *D. villosum,* whereas isoform 17 was detected in the populations of *L. elongatum* only. Comparison of the above mentioned species resulted in a value of 0.45 for both indices SI and S. Much higher values (SI = 0.77, S = 0.82) were obtained when *L. elongatum* was contrasted to *A. cristatum.*

Tab. 4. Mean isoform frequencies of cathodal peroxidase in the studied populations of *Agropyron cristatum, Dasypyrum villosum* and *Lophopyrum elongatum.* Each isoform was assigned a number which reflects its gel migration (in mm) from the origin.

Species	Isoforms										
	5	10	14	17	24	28	30	32	36	40	45
A. cristatum	0.00	0.66	0.28	0.00	0.58	0.00	0.74	0.00	0.42	0.60	0.22
D. villosum	0.45	1.00	0.55	0.00	0.00	1.00	0.00	0.92	1.00	0.71	0.00
L. elongatum	0.00	0.72	0.38	0.62	0.82	0.00	0.49	0.22	0.72	0.53	0.34

In total, twelve isoforms of ACP were found in the species examined (Tab. 5). Populations of *D. villosum* and *L. elongatum* contained three (6, 32, 38) and one (16) diagnostic isoform(s), respectively. The taxa exhibited a moderate level of similarity. Index SI ranged from 0.33 *(L. elongatum* vs. *D. villosum*) to 0.44 in the comparison between the former species and *A. cristatum.* Respective figures for index S varied in the range of 0.33 to 0.55.

Tab. 5. Mean isoform frequencies of acid phosphatase in the studied populations of *Agropyron cristatum, Dasypyrum villosum* and *Lophopyrum elongatum.* Each isoform was assigned a number which reflects its gel migration (in mm) from the origin.

Species	Isoforms											
	6	11	16	18	20	22	24	28	29	32	36	38
A. cristatum	0.00	1.00	0.00	1.00	0.00	0.75	0.00	0.20	0.28	0.00	0.42	0.00
D. villosum	0.96	1.00	0.00	1.00	0.39	0.89	1.00	0.89	0.00	0.22	0.00	0.78
L. elongatum	0.00	0.69	0.75	0.00	0.80	0.00	0.28	0.11	0.55	0.00	0.58	0.00

Mean values of SI in the comparison of *D. villosum* and the species pair *L. elongatum – A. cristatum* were 0.35 and 0.42, respectively. A mean SI value of 0.54 was obtained when the latter two species were contrasted. Although slightly higher, values of index S followed the same tendency. In contrast to the other three indices, higher values of index D mean lower affinities. Nearly identical mean values of D (0.56, 0.55) were obtained in the comparison of *D. villosum* with *L. elongatum* and *A. cristatu*m. A lower value (D = 0.46) was calculated when the latter two species were compared. The comparison of *A. cristatum* with the other two species resulted in a mean value of I_h equal to 0.49 in both cases. A lower value (I_h = 0.36) was calculated for comparison of *L. elongatum* and *D. villosum.*

Discussion

Isoenzyme variation of the enzymes EST, PER and ACP indicated that *A. cristatum, L. elongatum* and *D. villosum* are clearly differentiated entities since a relatively small portion of isoforms was shared by all species. Quantitative evaluation of their isoenzyme structure is also informative. All similarity indices used show the lowest affinity in the comparison between *L. elongatum* and *D. villosum.* This is an indication that the two species are most distantly related as judged by the enzymes surveyed. Although slightly higher, the relatively low values of SI, S and I_h show that *D. villosum* and *A. cristatum* are also remotely positioned. *Dasypyrum villosum* possessed the highest number of diagnostic isoforms that is an additional indicator of its distinctness within the group studied. The species *A. cristatum* and *L. elongatum* demonstrated a higher level of affinity as estimated by all the indices used.

The present results are consistent with those of MCINTYRE (1988) who has studied larger species sample within the tribe Triticeae. Phenetic and cladistic analysis separated the species into two groups. The first comprised P, V, H genome species. Within this group, the dichotomy between P and V genome species was greater than that found between any other species in the analysis. The second group comprised another dichotomy, the first containing S genome species and the second containing *L. elongatum* (E genome), *Triticum monococcum* (A genome) and J, JJ genome species. These findings indicate that although distantly related, *Dasypyrum* is closer to *Agropyron* than to *Lophopyrum*. More recent phylogenetic analysis of Triticeae based on morphology (SEBERG and FREDERIKSEN 2001) clustered *Dasypyrum* with *Crithodium (Triticum)* and *Secale* – a relationship inferred from crossing data. However, it should be pointed that a low degree of chromosome pairing between *Dasypyrum* and different species of *Triticum, Aegilops* and *Secale* has been shown (BLANCO et al. 1983, JAN et al. 1986, LUCAS and JAHIER 1988), thus indicating a weak genomic similarity between *Dasypyrum* and the above mentioned genera. Moreover, *D. villosum* differs from both wheat and rye by several isoenzymes (JAASKA 1975, 1982). It should be emphasized that morphological data are not reliable for phylogenetic reconstructions in the Triticeae as they exhibit a great deal of homoplasy (KELLOGG 1992). Starch synthase data (MASON-GAMER and KELLOGG 2000) suggested a paraphyletic origin for *Dasypyrum* and provided evidence of past gene exchange between the former genus and *Lophopyrum*. On the contrary, combined data from three nuclear genes (KELLOGG et al. 1996, KELLOGG 1998) placed *Dasypyrum* basally and distantly from both *Agropyron* and *Lophopyrum* but closer to the former genus.

Isoenzyme data presented here are mostly congruent with nuclear DNA data and do not support cpDNA data. The most likely genes encoding the enzymes assayed are nuclear ones and reflect a portion of nuclear genome. There is conflict between data derived from different genetic markers in the Triticeae. Nuclear gene trees are significantly different, both from each other and from chloroplast phylogeny (MASON-GAMER and KELLOGG 1996b). MASON-GAMER and KELLOGG (2000) suggest that discordance among different data sets reflects widespread reticulations among the intersterile diploid genera, existence of numerous allopolyploid genomic combinations and hybridization among polyploids. Several statistical tests applied (MASON-GAMER and KELLOGG 1996b) lead to the conclusion that nuclear and chloroplast DNA trees for the Triticeae reflect significantly different evolutionary histories.

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