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Research Article

# Isoenzyme Variation of Esterase and Acid Phosphatase and Genetic Affinities among *Dasypyrum villosum* (L.) P.Candargy, *Elytrigia repens* (L.) Nevski and *Elymus caninus* (L.) L.

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**Abstract:** Polyacrylamide gel electrophoresis was employed to study the isoenzyme variation of esterase and acid phosphatase in natural populations of *Dasypyrum villosum* (L.) P.Candargy, *Elytrigia repens* (L.) Nevski and *Elymus caninus* (L.) L. Four similarity indices (SI, S, D, $I_h$) were calculated in an attempt to evaluate quantitatively genetic affinities among the species examined. Considering index D, the species *D. villosum* proved to be equally distant (D = 0.17 in both cases) from the species pair *Et. repens* and *El. caninus*. The nearly twice lower value of D for the comparison between *Et. repens* and *El. caninus* is an indication of their stronger genetic relationship. Mean values of indices $I_h$, SI and S also indicated that *D. villosum* is the most distinct species within the group studied. The results were discussed in the light of chloroplast DNA sequence data, suggesting a close affinity among the genera *Dasypyrum* (Coss. & L.Durieu) T.Durand, *Elytrigia* Desv. and *Elymus* L. The results of the present isoenzyme study are not in congruence with cpDNA analysis. Both isoenzyme and DNA data suggest that the phylogenetic position of the genus *Dasypyrum* within the tribe *Triticeae* remains unresolved.

Key Words: *Dasypyrum villosum, Elytrigia repens, Elymus caninus*, esterase, acid phosphatase, isoenzyme variation, genetic affinities

## Introduction

*Dasypyrum* (Coss. & L.Durieu) T.Durand is a small genus which belongs to the subtribe *Triticinae* of the tribe *Triticeae* (Tzvelev, 1976). Two species of *Dasypyrum* are distributed in Europe: the perennial *Dasypyrum hordeaceum* (Coss. & L.Durieu) P.Candargy and the widespread annual *D. villosum* (L.) P.Candargy (Humphries, 1978). Both species are diploids. Morphologically, *Dasypirum* is considered to be closely related to *Triticum* L., *Agropyron* Gaertn. and *Secale* L.

Chloroplast DNA (cpDNA) restriction site diversity has been used to address a wide range of evolutionary problems. Recent studies of *Triticeae* based on molecular data (Kellogg, 1992a; Kellogg, 1992b; Mason-Gamer & Kellogg, 1996) suggested that a close phylogenetic relationship existed among *Dasypyrum, Elytrigia* Desv. *Elymus* L. at the DNA level.

In a previous analysis of several enzymes (unpubl. res.) it was demonstrated that the species *D. villosum* was clearly distant from both *Elytrigia repens* (L.) Nevski and *Elymus caninus* (L.) L.,, while the latter two species exhibited relatively little divergence at the isoenzyme level. The present paper extends the study of isoenzyme variation in natural populations of *D. villosum, Et. repens* and *El. caninus* by including two additional enzymes. The purpose was to contribute further understanding of the genetic affinities among these species and the respective genera by means of isoenzymes.

## Materials and Methods

The isoforms of enzyme esterase and acid phosphatase were analysed in 94 individual plants from three populations of *Et. repens,* 72 plants from two populations of *El. caninus* and 150 plants from four populations of *D. villosum* (Table 1). Vouchers are deposited at the herbarium of Institute of Botany (SOM).

Leaves were ground in 0.01 M Tris, 0.08 M. glycine, 0.005 M cysteine, and 20% sucrose at pH 8.3. Ion-exchange resin Dowex 1 x 8 (0.4 g / 1 g fresh tissue) was added to the extraction buffer to eliminate polyphenols. Homogenates were centrifuged at 10,000 rpm for 10 min. The supernatant was used as a source of enzymes.

Table 1. Species and populations examined.

| Species | Number of individuals | Locality | Voucher number |
|---|---|---|---|
| *Et. repens* | 33 | Vitosha Mt., around the village of Marchaevo | Co-597 |
| | 28 | Sredna gora Mt., near the village of Dushantsi | Co-598 |
| | 30 | Sredna gora Mt., in the surroundings of Pirdop | Co-599 |
| *El. caninus* | 35 | Rila Mt., the valley of Rilska river | Co-591 |
| | 11 | Estonia, Laelatu, EE 2003 | Co-421 |
| *D. villosum* | 40 | Chepan Mt., around Dragoman | Co-225 |
| | 35 | Strouma valley region, Kozuh hills | Co-226 |
| | 24 | Strouma valley region, near the village of Marikostinovo | Co-600 |
| | 41 | Thracian region, around the village of Levka | Co-228 |

Anodally migrating isoforms of esterase and acid phosphatase were resolved on 7.5% polyacrylamide slabs as separating gel with 3% stacking gel by the electrophoretic system of Davis (1964). Cathodal isoforms of EST were run on 7.5% separating gel and 3% stacking gel according to Reisfeld et al. (1961). The length of the separating gel was 6 cm and stacking gels were 1.5 cm long. Electrophoresis was conducted at 200 V/25 mA for the basic gels and at 150 V/45 mA for the acidic gel system. Electrophoresis of cathodal esterase 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 acid phosphatase. Staining protocols were performed as mentioned in Angelov (2000).

Knowledge of the subunit structure of the enzymes examined and the patterns of their segregation within natural populations did not facilitate genetic interpretation of enzyme phenotypes. The complex phenotypes observed made impossible the genetic determination of enzyme phenotypes. For this reason, two phenetic parameters were employed: 1) isoform (band) presence/absence and 2) isoform frequency. Each isoform was assigned a number reflecting its gel migration in mm from the origin (Perez de la Vega & Allard, 1984).

The phenotypic diversity of each species was measured in several ways: 1) the number of isoforms detected and 2) the polymorphic index (PI), which was calculated according to Singh and Jain (1971):

$$PI = \sum_{i=1}^{N} R_i\,(1-R_i)$$

where $R_i$ is the frequency of the ith isoform in a given species and N is the number of isoforms observed in the same species.

3) Specific polymorphic index $PI_S$ = PI/N was also calculated (Marshall & Jain, 1969).

Based on presence/absence data, the average values of two measures of phenetic affinity were calculated as follows:

1) Similarity index (SI) of Jaccard (see Chung et al., 1991)

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

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

2) Coefficient of similarity (S) of Sneath & Socal (after Kalinowski et al., 1979)

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

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

Average phenotypic identities among species examined were calculated by Hedrick's (1971) measure of phenotypic identity

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

where,

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

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

Additionally, the coefficient of differentiation (D) was calculated according to the following formula:

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

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

**Results and Discussion**

Totally nine isoforms of cathodal esterase were detected in the species studied (Table 2). Isoforms 13 and 18 were specific for *D. villosum*. Isoforms 34, 38 and 40 occurred in species pair *Et. repens* and *El. canin*us only. Indices SI and S varied in a wide range – from 0.33 (*D. villosum* vs. *Et. repens*) to 0.83 in the comparison between the latter species and *El. caninus*. The calculation of coefficient D resulted in values of 0.18 and 0.20 when comparing *D. villosum* with *Et. repens* and *El. caninus*, respectively.

The isoform frequencies of anodal esterase are shown in Table 3. Sixteen isoforms were electrophoretically detected. Four of them (isoforms 18, 23, 41 and 45) were invariant in *D. villosum.* Most of the isoforms were shared by all the species studied, but isoform 14 was diagnostic for *D. villosum* and isoforms 35 and 43 occurred in *Et. repens* and *El. caninus* only. Similarity indices SI and S ranged from 0.68 to 0.75. Coefficient D varied in the range from 0.09 for the comparison between *El. caninus* and *Et. repens* to 0.13 when the latter was compared with *D. villosum.*

Sixteen isoforms of acid phosphatase were detected (Table 4). Isoforms 6 and 18 were invariant and diagnostic for *D. villosum.* Isoforms 30 and 42 were specific for *Et. repens.* Index SI ranged from 0.35 (*D. villosum* vs. *Et. repens*) to 0.60 when the latter and *El. caninus* were compared. The calculation of coefficient D resulted in values of 0.19 and 0.17 when *D. villosum* was compared to *Et. repens* and *El. caninus.*

The species *Et. repens* and *El. caninus* had a greater number of isoforms (30 and 31), and a higher average PI per enzyme (1.73 and 1.39) and $Pi_S$ (0.14 and 0.13), respectively. There were 28 isoforms observed in *D. villosum.* It had the lowest average PI (0.77) and $Pi_S$ (0.07) values.

The average values of similarity index SI for the comparison of *D. villosum* with species pair *Et. repens* and *El. caninus* were 0.46. and 0.57, respectively. The corresponding value for the comparison between *Et. repens* and *El. caninus* was 0.71. Similar though slightly higher values of index S were obtained. The comparison of *D. villosum* with *Et. repens* and *El. caninus* resulted in average values of coefficient D equal to 0.17 in both cases, whereas an average value of 0.10 was calculated when the latter two species were compared. The values of phenetic identity measure $I_h$ were 0.33 and 0.42 when *D. villosum* was contrasted with *Et. repens* and *El. caninus*, whereas the comparison between the latter two species resulted in a value of 0.50.

Table 2. Average isoform frequencies of cathodal esterase in the studied populations of *Et. repens*, *El. caninus* and *D. villosum*.

| Species | Isoforms | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 13 | 18 | 25 | 30 | 34 | 38 | 40 | 42 |
| *Et. repens* | 0.00 | 0.00 | 0.22 | 0.28 | 0.22 | 0.22 | 0.17 | 0.00 |
| *El. caninus* | 0.00 | 0.00 | 0.08 | 0.05 | 0.08 | 0.15 | 0.55 | 0.09 |
| *D. villosum* | 0.06 | 0.56 | 0.56 | 1.00 | 0.00 | 0.00 | 0.00 | 1.00 |

Table 3. Average isoform frequencies of anodal esterase in the studied populations of *Et. repens, El. caninus* and *D. villosum*.

| Species | Isoforms | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 14 | 16 | 18 | 21 | 23 | 26 | 28 | 30 | 33 | 35 | 37 | 41 | 43 | 45 | 48 | 50 |
| *Et. repens* | 0.00 | 0.09 | 0.09 | 0.48 | 0.04 | 0.24 | 0.35 | 0.41 | 0.11 | 0.41 | 0.04 | 0.30 | 0.11 | 0.48 | 0.30 | 0.20 |
| *El. caninus* | 0.00 | 0.03 | 0.00 | 0.52 | 0.22 | 0.13 | 0.32 | 0.42 | 0.19 | 0.13 | 0.42 | 0.97 | 1.00 | 0.42 | 0.71 | 0.58 |
| *D. villosum* | 0.06 | 0.11 | 1.00 | 0.11 | 1.00 | 0.66 | 0.94 | 0.06 | 0.11 | 0.00 | 0.39 | 1.00 | 0.00 | 1.00 | 0.11 | 0.11 |

Table 4. Average isoform frequencies of acid phosphatase in the studied populations of *Et. repens, El. caninus* and *D. villosum.*

| Species | Isoforms | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6 | 11 | 14 | 16 | 18 | 20 | 22 | 23 | 24 | 26 | 28 | 30 | 32 | 36 | 38 | 42 |
| *Et. repens* | 0.00 | 0.25 | 0.57 | 1.00 | 0.00 | 0.28 | 0.43 | 0.43 | 0.28 | 0.00 | 0.00 | 0.57 | 0.28 | 1.00 | 0.00 | 0.57 |
| *El. caninus* | 0.00 | 0.75 | 0.90 | 1.00 | 0.00 | 0.63 | 0.33 | 0.16 | 0.53 | 0.10 | 0.95 | 0.00 | 0.00 | 1.00 | 0.79 | 0.00 |
| *D. villosum* | 1.00 | 1.00 | 0.00 | 0.00 | 1.00 | 0.39 | 0.89 | 0.00 | 0.94 | 0.00 | 0.94 | 0.00 | 0.11 | 0.00 | 0.89 | 0.00 |

All phenetic parameters for enzymes esterase and acid phosphatase revealed similar patterns of genetic relationships among the species.

Considering coefficient D, the species *D. villosum* proved to be equally distant (D = 0.17 in both cases) from the species pair *Et. repens* and *El. caninus*. This value of D indicates that a substantial genetic differentiation exists between *D. villosum* and the latter two species. The nearly twice lower value of coefficient D for the comparison between *Et. repens* and *El. caninus* is an indication of their stronger genetic relationship. The mean values of $I_h$ also indicated, although not so definitely, that *D. villosum* is the most distinct species within the group studied. Similarity indices SI and S also supported the observation that a closer genetic affinity exists between the latter two species, whereas *D. villosum* is the most distantly positioned within the studied group of *Triticeae*. Considering together all phenetic parameters, it could be concluded that *Et. repens* and *El. caninus* are genetically more closely related than either is to *D. villosum*. The latter species proved to be clearly differentiated at the genes coding for the set of soluble enzymes surveyed.

Chloroplast DNA (cpDNA) restriction site variation has been used to generate phylogenetic trees of monogenomic genera within the tribe *Triticeae* (Kellogg, 1992b). The most distinctive molecular marker was a unique deletion found in *D. villosum, Pseudoroegneria libanotica* (Hackel) Dewey *(Elytrigia libanotica* (Hackel) Holub*)* and *Ps. stipifolia* (Chern. ex Nevski) A.Löve *(Et. stipifolia* (Chern. ex Nevski) Nevski). The deletion was first detected in *Et. repens* (Kellogg, 1992a). Later, Mason-Gamer and Kellogg (1996) demonstrated that polyploids of *Elymus* L. and *Elytrigia* Desv. formed a moderately well supported clade with *Dasypyrum* (Coss. & Durieu) and *Pseudoroegneria* (Nevski) A.Löve. The latter genus, as well as *Elytrigia* and *Elymus,* contains the S genome. Thus, the deletion may be a useful marker for the S genome but it will not distinguish the S genome from the V genome of *D. villosum.* Although cpDNA data indicated a strong affinity between *Dasypyrum* and *Pseudoroegneria* chloroplast genomes, the two groups appeared to be distant on the basis of morphological data (Kellogg, 1989).

Some phylogenetic reconstructions based on morphology grouped *D. villosum* with *Crithodium monococcum* (L.) A.Löve *(Triticum monococcum* L.) and *Secale cereale* L. (Seberg & Frederiksen, 2001), but morphological trees are very unstable and exhibit a great deal of homoplasy (Kellogg, 1992a; Frederiksen & Seberg, 1992). Hence, it seems difficult to determine the phylogenetic position of *Dasypyrum* on the basis of morphology. Moreover, it has been demonstrated that the species *D. villosum* differs from both wheat and rye for a number of isoenzyme loci (Jaaska, 1975, 1982).

Genomic relationships in the tribe *Triticeae* have been investigated in a series of studies (McIntyre, 1988; McIntyre et al., 1988a, 1988b; Scoles et al., 1988) by means of morphology, chromosome pairing, isoenzymes, DNA hybridization and sequencing. The relative position of the V genome varied between analyses depending on the parameters employed. In general, it exhibited affinity to the S, E and J genomes (McIntyre, 1988). These findings correspond partially to cpDNA restriction site variation studies. Both approaches indicate that an affinity between the V genome species *D. villosum* and the S genome species pair *Et. repens* and *El. caninus* exists, at least, for a portion of their genomes.

The results of the present study of *D. vilosum, Et. repens* and *El. caninus* are not in congruence with cpDNA analysis. It was demonstrated that the former species is genetically distinct from both *Et. repens* and *El. caninus*, as revealed by the isoenzymes of esterase and acid phosphatase. Both isoenzyme and DNA data (Kellogg et al., 1996, Kellogg, 1998; Kellogg, pers. comm.) suggest that the phylogenetic position of the genus *Dasypyrum* within the tribe *Triticeae* remains unresolved. Mason-Gamer and Kellogg (1996) compared statistically four sets of molecular data to determine whether they were significantly different. It was concluded that the cpDNA data set reflects an evolutionary history substantially different from that of any nuclear DNA data sets. The cause of this discrepancy between chloroplast and nuclear genomes remains unknown.

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