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Isoenzyme variation and genetic relationships among *Elytrigia junceiformis, E.* × *littorea* and *E. repens* (Triticeae: Poaceae)

Georgi B. Angelov

Institute of Botany, Bulgarian Academy of Sciences, ul. Acad. G. Bonchev, bl. 23., BG-1113 Sofia, Bulgaria (e-mail: gbangv@iph.bio.bas.bg)

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Electrophoresis of eleven enzymes was employed to study isoenzyme variation and genetic affinities among the hybrid *Elytrigia* × *littorea* and its parents *E. repens* and *E. junceiformis*. *Elytrigia* × *littorea* shared most of isoenzyme profiles and isoforms of its parents but no evidence for a strict additiveness was observed. The isoenzyme structure of the hybrid resembled more the structure of *E. repens*. Two alternative interpretations for this are discussed. The exact genome composition of *E.* × *littorea* should be studied to confirm which interpretation of the isoenzyme data is correct.

Key words: *Elytrigia junceiformis, E.* × *littorea, E. repens*, genetic relationships, isoenzyme variation, Poaceae, Triticeae

Introduction

Elytrigia junceiformis A.&D. Löve belongs to the *Junceum* polyploid complex, which includes perennial littoral taxa from diploids to decaploids (Melderis 1978) with a predominantly rhizomatous pattern of growth. *Elytrigia junceiformis* is a coastal species and grows on the shores of the North Atlantic and the Baltic Sea. The taxa of the *Junceum* polyploid complex have been treated as members of different grass genera — *Agropyron, Elymus,* and *Thinopyrum*. Thus, *Agropyron junceiforme* A.&D. Löve, *A. junceum* ssp. *boreo-atlanticum* Simon. & Guinoch., *Elymus farctus* (Viv.) Runemark *ex* Melderis ssp. *boreali-atlanticus* (Simon. & Guinoch.) Melderis, and *Thinopyrum junceiforme* (A.&D. Löve) D. Löve are among the common synonyms of *E. junceiformis*.

Elytrigia repens (L.) Nevski has also been placed in *Agropyron* and *Elymus* under the synonymous names *Agropyron repens* (L.) Beauv. and *Elymus repens* (L.) Gould., respectively. Spontaneous natural hybrids between species of the *Junceum* polyploid complex and *Elytrigia repens* have been repeatedly reported (Östergren 1940, Godley 1951, Heneen 1963). More recently it has been shown that *E. repens* as an outcrossing species often hybridizes with the *Thinopyrum junceum* group (*JJJ* and *JJ* genomes) and *T. intermedium* (*JJS* genomes) in the field (Melderis 1980, Assadi & Runemark 1995). Artificial hybrids of *E. junceiformis* have also been produced (McGuire 1984, Piennar *et*

al. 1988, Wang & Hsiao 1989).

Elytrigia × *littorea* (Schum.) Hyl. is a natural hybrid of *E. junceiformis* and *E. repens* and it grows on the shores of the Baltic Sea.

Electrophoretic analysis of isozymes has frequently been used to shed light on phylogenetic relationships among different taxa within Triticeae (Jaaska 1972, 1982, Jaaska & Jaaska 1982, 1984, Jörgensen 1986, McIntyre 1988). Seed protein electrophoresis proved to be an efficient method for assessing systematic relationships among different taxa of polyploid series like the *Junceum* complex (Moustakas & Coucoli 1982, Symeonidis *et al*. 1985, Moustakas *et al*. 1986, Moustakas *et al*. 1988). To my knowledge no electrophoretic study of hybrid *Elytrigia* × *littorea* and its parents has been conducted so far.

In this paper electrophoretic data for several natural populations of *Elytrigia junceiformis*, *E. repens* and *E.* × *littorea* are reported. The aim of this study was to shed light on the genetic relationships among the hybrid and its parents by means of isoenzymes.

Material and methods

Nine to thirty four plants from natural populations (Table 1) were collected randomly and maintained in a greenhouse at the Institute of Botany. Vouchers were deposited at the Institute of Botany Herbarium (SOM).

Individual samples for isoenzyme analyses were taken from the living collection. Leaves were ground in 0.01 M Tris, 0.08 M glycine, 0.005 M cysteine, 20% sucrose, pH 8.3. Ion-exchange resin Dowex 1 × 8 (0.4 g l^{-1} g tissue) was added to the extraction buffer to bind polyphenols. Crude extracts were centrifuged at 10 000 rpm for 10 minutes. The supernatant was used as a source of enzymes. Eleven enzymes, namely esterase (EST), peroxidase (PER), acid phosphatase (ACPH), aspartate aminotransferase (AAT), superoxide dismutase (SOD), amylase (AMY), tetrazolium oxidase (TO), NADH-diaphorase (DIA), catalase (CAT), glucose-6-phosphate dehydrogenase (G-6-PDH), glutamate dehydrogenase (GDH) were used as molecular markers. Anodal isoforms, without PER, were assayed by discontinuous polyacryamide gel electrophoresis (PAGE) system, pH 8.3 (Davis 1964). Vertical slabs with 7.5% separating and 3% stacking gels were employed. The cathodal isoforms of EST, PER and ACPH were resolved by the acidic (pH 4.3) PAGE system of Reisfeld *et al*. (1961). Electrophoresis was conducted at 200 V/25 mA for basic gels and at 150 V/45 mA for acidic gel system. The length of the separating gel for cathodal EST, PER, ACPH, SOD, anodal EST was 6 cm and 5 cm for the rest of enzymes studied. Electrophoresis in anodal direction was carried out until the indicator dye, bromphenol blue, reached the gel end (1 front) for enzymes TO, SOD, GDH, 1.25 fronts for EST and 1.5 fronts for the rest of enzymes. Electrophoresis in cathodal direction was performed until the tracking dye, pyronin G, reached the end for EST and ACPH and 1.5 fronts for PER. The isoforms of AMY were fractionated on 6% separating gels containing 0.5% starch for 12 h at 150 V. All procedures were carried out at 4 °C.

Staining protocols for EST, ACP, AAT, GDH, G-6-PDH were previously described (Angelov 2000). Staining recipes for AMY and PER (Przy-

Table 1. Taxa and populations studied.

Taxon	Locality	Voucher
E. repens	Bulgaria, Sredna Gora Mt., the village Anton	Co-435
	Bulgaria, Sofia region, the village Lozen	Co-436
	Bulgaria, Strouma valley, near the town Simitli	Co-437
	Estonia, the town Tartu, Maarjamõisa	Co-438
	Estonia, the southern vicinities of Tartu, Ränilinn	Co-439
	Estonia, Kääriku	Co-440
E. × *littorea*	Estonia, Saaremaa, Murika	Co-441
E. junceiformis	Estonia, Saaremaa, Harilaid	Co-442
	Estonia, Hiiumaa, Kalana	Co-443

bylska *et al*. 1982), CAT (Woodbury *et al*. 1970), SOD (Jaaska & Jaaska 1982) and DIA (Wendel & Veeden 1989) were slightly modified. TO was visualized according to Baur and Schorr (1969). Each isoform was assigned a number which reflects its gel migration in mm from the origin (Shumaker & Babble 1980, Perez de la Vega & Allard 1984).

The studied taxa are auto/allopolyploids and produce complex band patterns difficult to be interpreted genetically. For this reason, phenotypic analysis (isoform presence/absence and isoform frequency) was preferred instead of genotypic one (allele and genotype frequencies) to assess affinities among the taxa examined. Similar approaches have been successfully employed by Meerow (1987, 1989) for *Eucharis*, Chung *et al*. (1991) for *Hosta*, and Wilson (1999) and Samman *et al*. (2000) for *Festuca*. Thus, mean values of three measures of phenetic affinity, namely, S_J (Jaccard Similarity Coefficient, *see* Stuessy 1990), S_{SM} (Simple Matching Coefficient, Sokal & Michener 1958, *see* Stuessy 1990) and D_{CD} (Coefficient of Divergence, Clark 1952, *see* Stuessy 1990) for the six most polymorphic enzymes EST, ACPH, PER, DIA, SOD, TO were calculated from presence/absence and isoform frequency data according to the following formulas:

$$S_J = a/(a + b + c), \quad (1)$$

where a is the number of isoforms common for both taxa, and b and c are the numbers of isoforms specific for each taxa compared,

$$S_{SM} = (a + d)/(a + b + c + d), \quad (2)$$

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

$$D_{CD} = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(x_{ij} - x_{ik}\right)^2} \quad (3)$$

where N is the total number of isoforms for each enzyme, and x_{ij} and x_{ik} are the frequencies of i-th isoform in taxa j and k.

Results

Catalase. Two isoforms (11, 12) were found in the populations studied. The Bulgarian populations of *Elytrigia repens* (hereafter referred to as *E. repens* BG) were invariant in respect to isoform 12 while isoform 11 was fixed in *E. junceiformis*. Isoform 11 prevailed in *E.* × *littorea* and both isoforms were found in Estonian (EE) populations of *E. repens*.

Glutamate dehydrogenase. The isoform 13 was monomorphic for *Elytrigia* × *littorea* and nearly monomorphic for *E. junceiformis*. The same isoform dominated in *E. repens* EE and *E. repens* BG but they differed as the former possessed also isoform 12, while the latter possessed additionally isoform 14.

Glucose-6-phosphate dehydrogenase. Triplet 23/26/29 was fixed in *Elytrigia repens* and was shared by *E.* × *littorea,* while another triplet (23/24.5/26) was characteristic of the latter. Two types of triplets (17/20/23 and 20/23/26) clearly distinguished *E. junceiformis* from *E. repens* and *E.* × *littorea*. Different types of triplets found in the *Elytrigia* taxa examined reflect dimeric subunit structure of G-6-PDH generally observed in plants (Weeden & Wendel 1989).

Aspartate aminotransferase. Numbered from the anode, three zones of independent isoenzyme variation were detected on the gels. Most probably they reflect the exsistence of three gene loci for AAT in the *Elytrigia* species. The isoform 20 in zone III was invariant in all species. *Elytrigia* × *littorea* and *E. repens* shared isoform 29 in zone II. The triplet 31/33/35 in zone I was fixed in *E. junceiformis*. The same triplet was also rarely observed in *E. repens* EE. The occurrence of triplets indicates dimeric structure of AAT as it has been generally found in plant species (Weeden & Wendel 1989).

Amylase. Two zones of independent variation were observed, suggesting two gene loci of AMY. *Elytrigia junceiformis* was invariant for isoform 9 in the slower migrating zone. *Elytrigia* × *littorea* was nearly fixed for the same isoform. Due to the presence of doublets 9/11 and 10/11 in *E. repens*, the former two species could be distinguished from both EE and BG populations of *E. repens*. All species studied were monomorphic for isoform 28 in the faster migrating zone of AMY.

Superoxide dismutase. The isoenzyme variation of SOD is shown in Table 2. Two isoforms, 25 and 39, were monomorphic in all taxa. Isoform 36 was observed only in Estonian populations of *Elytrigia repens*. The triplet 35/37/39, indicating dimeric structure of SOD (Jaaska & Jaaska 1982, Weeden & Wendel 1989), was shared by *E.* × *littorea* and *E. junceiformis*. Thus, the latter two species were clearly differentiated from *E. repens* in regard to SOD.

Cathodal acid phosphatase. The isoform 25 was shared by all taxa investigated. Isoform 20 was shared by *Elytrigia* × *littorea* and *E. repens*, while isoform 32 was specific for the latter.

Anodal acid phosphatase. In total, ten isoforms were electrophoretically resolved (Table 3). Isoforms 24 and 30 were monomorphic in all species. *Elytrigia* × *littorea* carried two unique isoforms, 20 and 22, which were not observed in its parents. *Elytrigia junceiformis* also possessed two specific isoforms, 16 and 32, which clearly distinguished it from *Elytrigia* × *littorea* and *E. repens*.

Tetrazolium oxidase. Although TO is considered as a synonym of SOD (Beauchamp & Fridovich 1973), the staining recipes used in this study differed and produced different electrophoretic patterns. Hence, we think that TO could be used as another isoenzyme marker of the present phenetic analysis (Table 4). Three isoforms, 29, 32 and 45, were fixed in all taxa, while another set of isoforms (9, 13, 18) was invariant in *Elytrigia* × *littorea* and Bulgarian populations of *E. repens*. The isoform 26 was found only in *E.* × *littorea*.

Diaphorase. Totally seven isoforms were resolved (Table 5). Most of them were monomorphic in two or all three species. Isoform 37

Table 2. Variation of superoxide dismutase isoenzymes in *Elytrigia repens*, *E. junceiformis* and *E.* × *littorea*.

Taxon	Isoform frequency						
	18	22	25	35	36	37	39
E. repens EE	0.67	0.33	1.00	0.00	0.50	0.00	1.00
E. repens BG	1.00	0.00	1.00	0.00	0.00	0.00	1.00
E. junceiformis	1.00	0.00	1.00	0.16	0.00	0.25	1.00
E. × *littorea*	0.25	0.75	1.00	0.25	0.00	0.25	1.00

Table 3. Variation of anodal acid phosphatase isoenzymes in *Elytrigia repens*, *E. junceiformis* and *E.* × *littorea*.

Taxon	Isoform frequency									
	11	14	16	18	20	22	24	30	32	36
E. repens EE	0.45	0.28	0.00	0.86	0.00	0.00	1.00	1.00	0.00	1.00
E. repens BG	0.50	0.43	0.00	0.50	0.00	0.00	1.00	1.00	0.00	1.00
E. junceiformis	0.55	0.00	1.00	0.00	0.00	0.00	1.00	1.00	0.33	0.27
E. × *littorea*	0.67	0.67	0.00	0.66	0.34	0.33	1.00	1.00	0.00	0.18

Table 4. Variation of tetrazolium oxidase isoenzymes in *Elytrigia repens*, *E. junceiformis* and *E.* × *littorea*.

Taxon	Isoform frequency						
	9	13	18	26	29	32	45
E. repens EE	0.86	0.86	0.86	0.00	1.00	1.00	1.00
E. repens BG	1.00	1.00	1.00	0.00	1.00	1.00	1.00
E. junceiformis	0.00	0.00	0.00	0.00	1.00	1.00	1.00
E. × *littorea*	1.00	1.00	1.00	0.33	1.00	1.00	1.00

clearly distinguished *Elytrigia repens* from both *E. junceiformis* and *E.* × *littorea*. *Elytrigia junceiformis* carried the invariant, unique isoforms 21 and 41, not found in *E. repens* and *E.* × *littorea*.

Cathodal peroxidase. In total, eleven isoforms of the enzyme were resolved. Isoforms 10 and 29 were fixed across the whole group, while another set of isoforms (5, 27, 32, 37, 40, 45) was nearly fixed in most populations and taxa. The invariant isoform 35 was specific for *E. junceiformis*. Another monomorphic isoform 17 was observed only in *E.* × *littorea*.

Cathodal esterase. Two isoforms, 34 and 40, were monomorphic in all taxa studied. The isoform 25 was invariant in *Elytrigia repens* and it was nearly fixed in *E.* × *littorea* and *E. junceiformis*. The isoform 58 was shared by the latter two species and it distinguished them from *E. repens*.

Anodal esterase. In total, ten isoforms were detected (Table 6). Two invariant isoforms, 45 and 48, of *Elytrigia repens* clearly separated the former species from *E. junceiformis* and *E.* × *littorea*. Isoform 37 occurred in *E. junceiformis* only, while isoform 28 proved to be specific for *E.* × *littorea*.

The phenetic analysis of presence/absence (coefficients S_J and S_{SM}) and isoform frequency data (coefficient D_{CD}) allowed a more precise quantitative estimation of genetic affinities among the taxa. Mean values of S_J calculated for the six most polymorphic enzymes (EST, ACPH, PER, DIA, SOD, TO) are presented in Table 7. As one might expect, the Bulgarian and Estonian populations of *Elytrigia repens* demonstrated the closest affinity. *Elytrigia junceiformis* was clearly distinct from *E. repens*. The most striking observation was that *E.* × *littorea* was closer to *E. repens* than to *E. junceiformis*. The coefficient S_{SM}, with its stronger emphasis on double negative matches in pairwise comparisons, supported the S_J data. The Estonian and Bulgarian populations of *E. repens* differed slightly in their isoenzyme structure (S_{SM} = 0.93), while *E. junceiformis* had a substantially different structure (S_{SM} = 0.56–0.57). *Elytrigia* × *littorea* showed closer affinity to *E. repens* (S_{SM} = 0.72) and was more distant (S_{SM} = 0.62) from *E. junceiformis*. To the contrary of S_J and S_{SM}, greater values of D_{CM} mean lower affinities, e.g. D_{CM} = 0 in the case of identical set of isoforms occuring with identical frequences in taxa/populations under comparison, whereas the opposite extreme D_{CM} = 1 means a total lack of affinity. The mean values of coefficient D_{CM} are shown in Table 8. Regarding affinities among the species, similar tendencies are observed. The comparison

Table 5. Variation of diaphorase isoenzymes in *Elytrigia repens*, *E. junceiformis* and *E.* × *littorea*.

Taxon	Isoform frequency						
	21	25	27	30	33	37	41
E. repens EE	0.00	1.00	0.84	0.42	0.70	1.00	0.00
E. repens BG	0.00	1.00	1.00	1.00	1.00	1.00	0.00
E. junceiformis	1.00	0.00	1.00	0.40	0.40	0.00	1.00
E. × *littorea*	0.00	1.00	1.00	1.00	1.00	0.00	0.00

Table 6. Variation of anodal esterase isoenzymes in *Elytrigia repens*, *E. junceiformis* and *E.* × *littorea*.

Taxon	Isoform frequency									
	21	23	28	30	35	37	41	43	45	48
E. repens EE	0.00	0.75	0.00	0.25	0.00	0.00	0.40	0.75	1.00	1.00
E. repens BG	0.50	0.50	0.00	0.00	0.00	0.00	0.50	0.50	1.00	1.00
E. junceiformis	1.00	1.00	0.00	1.00	1.00	0.40	0.00	1.00	0.00	0.00
E. × *littorea*	0.00	1.00	0.35	0.33	0.35	0.00	0.65	1.00	0.00	0.00

between the Estonian and Bulgarian populations of *E. repens* resulted in the lowest value of D_{CM} (0.17) and indicated their closest affinity. The isoenzyme structure of the hybrid *E.* × *littorea* resembled more that of *E. repens*. At the same time *E. junceiformis* proved to be the most distant species among the studied taxa.

Summarizing the results, it was evident that *Elytrigia* × *littorea* is closer to *E. repens* for most of the examined enzymes (EST, ACPH, PER, DIA, TO, AAT). A smaller set of enzymes (AMY, SOD, GDH) indicated a closer affinity between the hybrid and *E. junceiformis*.

Discussion

Studies of *Tragopogon* (Roose & Gottlieb 1976) clearly demonstrated additive expression of some isoenzyme loci in hybrids of allopolyploid origin. In the present study of *Elytrigia* × *littorea, E. repens* and *E. junceiformis,* it was found that the hybrid shared most of the isoenzyme profiles and isoforms of its parents, but no evidence for a strict additiveness was observed. Similarly, studies of triticale (Rozynek *et al.* 1998) demonstrated that numerous samples showed gliadin band patterns different from the corresponding patterns of their parents. The results were explained by the hypothesis that wheat and rye genomes interact in triticale and the higher dose of wheat genome can suppress the expression of some secalin genes coded by the rye genome. Harsch *et al.* (1997) observed in *Triticum spelta* that some spelt cross-breads lack gliadin bands which are presented in the parental generation. The authors explained the complete absence of bands in the gliadin band patterns by the regulatory effects between the genomes of both cross parents.

Elytrigia junceiformis is a tetraploid species with 28 chromosomes (Östergren 1940). According to Löve's (1984) genomic system of classification, its genome constitution is *JJJJ*, if the species is considered as a strict autotetraploid. Other researchers (Moustakas *et al.* 1986, Moustakas *et al.* 1988, Jarvie & Barkworth 1990, Zhang & Dvorak 1990) found that the two genomes of *E. junceiformis* are not completely, but only nearly identical, with some slight differences still existing between them. Genome analyses (meiotic pairing patterns in hybrids, mitotic chromosome caryotypes) demonstrated that *E. junceiformis* is a segmental allotetraploid (Piennar *et al.* 1988, Liu & Wang 1992, Moustakas 1992) with two nearly identical *J* genomes (J_1 and J_2). *Elytrigia repens* is a allohexaploid (2n = 42) with a genome constitution SSSSHH (Assadi & Runemark 1995). The hybrid *E.* × *littorea* is a sterile pentaploid (2n = 35) and reproduces asexually by means of long rhizomes. Given the genome constitution of its parents, the genome formula of *E.* × *littorea* should theoretically be *SSHJJ* or $SSHJ_1J_2$, in the case of identical or nearly identical *J* genomes, respectively. Allopolyploids between *E* and *S* genomes showed isoenzyme patterns resembling those of *Pseudoroegneria* (*S* genome) species, thus reflecting patterns of variation shown by morphological and anatomical data (Jarvie & Barkworth 1990). In other studies of intergeneric crosses (Assadi & Runemark 1995) it was demonstrated that the *S* genome has almost always a dominance on the morphology of the taxa of which it is a component. Isozyme analysis of polyploids provided evidence of gene silencing through mutational or deletional processes and intergenomic suppression of redundant genes of polyploids (Galili & Feldman 1984, Wendel 2000). For example, the isoenzyme patterns of LAP in tetraploid *Chenopodium* species reflected that found in its diploid parental species. Thus loss of duplicated gene expression was proved to exist in *Chenopodium* (Wilson *et*

Table 7. Mean values of coefficient S_J for pair-wise comparisons among the taxa studied

Taxon	1	2	3	4
1 *E. repens* EE	×			
2 *E. repens* BG	0.89	×		
3 *E. junceiformis*	0.53	0.54	×	
4 *E.* × *littorea*	0.71	0.69	0.60	×

Table 8. Mean values of coefficient D_{CM} for pair-wise comparisons among the taxa studied

Taxon	1	2	3	4
1 *E. repens* EE	×			
2 *E. repens* BG	0.17	×		
3 *E. junceiformis*	0.62	0.53	×	
4 *E.* × *littorea*	0.42	0.39	0.48	×

al. 1983). A more recent DNA sequence analysis showed gene silencing in several polyploid species (Soltis & Soltis 1993).

The results of the present study could be interpreted in the light of the above-mentioned evidence about differential gene expression and silencing in polyploids. *Elytrigia* × *littorea* combines three (*S, H, J*) or four (*S, H,* J_1, J_2) different genomes. It could be assumed that the S genome dominates over the *J* genome and that some isoenzyme loci/alleles coded by the latter are suppressed or silenced in the hybrid species. In the classical example of *Tragopogon* (Roose & Gottlieb 1976), the derivative allotetraploid species is of very recent origin. It appeared in the early 1900s after the introduction of the diploid progenitors from the Old World (Novak *et al*. 1991), and it could be assumed that the genomes of its diploid parents have not changed substantially after the hybridization event. Electrophoretic studies also demonstrated that both copies of a duplicated gene usually retain expression in young polyploids (Gottlieb 1982), while in older polyploids loss of duplicated gene expression is common (Wendel 2000). Hence, in young allotetraploid species of *Tragopogon* electrophoretic profiles of its enzymes reflected exactly the summation of parental genomes. In the case of *E.* × *littorea,* where several different genomes interact, it could hardly be expected that the isoenzyme profiles would reflect exactly its genome composition. In addition, four isoforms of anodal ACP, DIA, TO and cathodal PER found in *E.* × *littorea* were not observed in its parents *E. repens* and *E. junceiformis*. One possible explanation is that *E.* × *littorea* has a more ancient origin, and other species carrying more or less different genomes may be involved in its parentage as was proposed by Aiken *et al*. (1993) for some species of the genus *Festuca*.

Alternatively, the observed nonadditivity of isoenzymes characteristic of the two parental species could be explained by the assumption that the analyzed specimens of *Elytrigia* × *littorea* are not strict allopolyploids, but segmental allopentaploids originated from a backcross by the *E. repens* pollen to the sterile F_1 hybrid between *E. repens* and *E. junceiformis*. In that case, one would expect a greater contribution by the *E. repens* parent to the pentaploid genome of *E.* × *littorea*. A study of the exact genome composition of *E.* × *littorea* is needed to show which one of the two alternative intrepretations of the isoenzyme data is correct.

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