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Halophile wheatgrass *Thinopyrum elongatum* (Host) D.R. Dewey (*Poaceae*) in three Apulian coastal wetlands: vegetation survey and genetic diversity

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Abstract

Three Apulian (SE Italy) coastal sites, Torre Canne to Torre San Leonardo (T), Punta della Contessa Salt Pans (S) and the La Vela Swamp (P), characterized by sandy shores and salt marshes recognized as wetland environments belonging to the Natura 2000 network, were selected as study areas in order to survey the vegetation zonation, plant communities, ecological context and genetic diversity of *Thinopyrum elongatum* population within humid habitats

Analysis ofvegetation, plant communities and soil samplings were carried out to investigate the ecological context of 24 populations collected of this halophile wheatgrass, along with biochemical characterization (A-PAGE, SDS-PAGE, and IEFxSDS-PAGE) of seed storage proteins, used to assess the genetic diversity intra- and inter-populations and genotype frequencies.

Thinopyrum elongatum populations showed a significative presence in the halophile shrub plant communities of salt marshes, especially those characterized by *Limbarda crithmoides* and *Artemisia coerulescens*. The differences of the protein patterns, suggest the presence in T area of a higher genetic variability and a greater resistance to the numerous factors of human pressure. The results have been discussed hypothesizing that this multidisciplinary approach could be considered a model to evaluate in the future the same environments or other similar ones, subjected to anthropogenic pressures and environmental changes.

Keywords: coastal wetland; Thinopyrum elongatum; vegetation analysis; ecological context; seed storage proteins; genetic diversity

Introduction

The genus *Thinopyrum*, within the *Poaceae* family, includes many halophytes, often cited with one or more taxonomic synonyms according to different adopted classification systems (Wang R. C. 2011). These halophytes are well adapted to sub-humid and arid climatic conditions, as those occurring in coastal environments where they contribute to some crucial functions such as environmental stabilization and coastal protection (Doing 1985; Acosta et al. 2007). Data concerning the genomic formula reports that the genus consists of diploids, segmental allotetraploids and allohexaploids, octaploids and decaploids species which possess the J-(or E)-genome, which Dewey (1984) designated as "J=E" and sometimes contains the St-genome.

The species *Thinopyrum elongatum* (Host) D.R. Dewey (common name: tall wheatgrass, 2n=14), also referred to as *Agropyron elongatum* (Host) P. Beauv., *Elytrigia elongata* (Host) Nevski, *Elymus elongatus* (Host) Runemark ssp. *elongatus*, *Lophopyrum elongatum* (Host) A. Löve, is herbaceous, cespitose, diploid species mainly distributed in the Eastern and southern Europe, particularly in the Mediterranean region, Caucasus, western Asia and northern Africa. The genus, the species and in general the synonyms of *Thinopyrum elongatum* at different ploidy levels, are well known and studied for tolerance to abiotic stresses such as drought, cold and salinity (Dvorak et al. 1988), resistance to pathogens, leaf and yellow rust, (Sarma & Knott 1966; Sharma et al. 1989; Ma et al. 2000; Shen et al. 2004; Hu et al. 2011), protein content and seed storage proteins (Dvorak et al. 1986; Li et al. 2008; Zhang et al. 2014).

All these traits, controlled by many genes located on different chromosomes, are relevant for cereal improvement however they are difficult to transfer in cultivated wheats even by chromosome engineering (Wang R.C. 2011; Ceoloni et al. 2014). Nevertheless, a large set of common wheat-*Thinopyrum elongatum* cytogenetic materials have been developed and evaluated for their agronomic performance (Dvorak & Knott 1974; Dvorak 1980; Tuleen & Hart 1988; Fu et al. 2012; Hayes et al 2012; Gazza et al., 2016; Kumar et al. 2017; Lou et al. 2017; Nie et al. 2019; Wang et al. 2010; Tanaka et al. 2017).

Thinopyrum elongatum (Host) D.R. Dewey usually thrives on saline soils of wetlands, and as a salt-tolerant and xerophilous grass, it is adapted to strong environmental stresses. Specific vegetation zonation and plant community successions characterize these humid habitats considered "dynamic", due to the action of several factors such as wind, waves, tides, and human activities, and essential for the conservation and restoration (Doing, 1985) of coast as well as for the identification of useful marker tracing biotic and abiotic environmental stresses (Colmer et al. 2006; Karan et al. 2012; Kubra et al. 2015). Moreover, in recent decades, wetland environments have undergone rapid anthropic development. In fact, increased human activity has resulted in biodiversity loss, habitat degradation reduction and fragmentation (Gray, 1997; Gibbs, 2000; Valdemoro et al. 2007, Tomaselli et al. 2012; Adamo et al., 2016), all these negative aspects associated with areas of particular interest characterized by high biological diversity and vulnerability.

In these environments, several authors have surveyed coastal plant communities in order to provide useful tools for habitat monitoring, plan strategic measures for the management or preservation of biodiversity, evaluation of cultural and economic value of humid areas (Van Der Maarel et al. 2003; Levin et al. 2009; Bunce et al., 2013; Tomaselli et al., 2017).

The dynamics of these coastal environments are also strictly associated to plant–plant interactions, which play a key role in community dynamics, in terms of substrate stabilisation and species successional replacement. Some species, as those belonging to the genus *Thinopyrum*, are particularly involved in such dynamics that can often change the genetic structure and geographic distribution of a whole population.

The evaluations of the genetic diversity of plant populations and species, genetic structure, geographic distribution in habitats or ecosystems under severe biotic and abiotic stresses, assessed by different approaches, in particular biochemical and molecular, have been often considered valuable tools to study evolutionary processes (Hughes et al. 2008). In the *Poaceae* grass family, markers of genetic variability of plant population useful to identify the link between habitat and species, ecotypes or plant genotypes with superior qualitative traits, are the seed storage proteins, gliadins and glutenins. Given that *Thinopyrum* is a distant wild relative of cultivated wheat (*Triticum aestivum* L), genotypes in this genus are a potential germplasm resource to study biodiversity changing in coastal habitat and new gliadin and

glutenin genetic variants represent a new genetic source to improve wheat quality and agronomic performance of cultivated wheats.

Concerning the seed storage proteins, gliadins and glutenins are respectively monomeric and polymeric proteins. Gliadins are fractionated by gel electrophoresis, at low pH in aluminium lactate buffer (A-PAGE), into α, β, γ and ω-gliadin (Payne et al. 1982). The *Gli-1* loci on the short arm of homoeologous group-1 chromosomes encode for all ω-gliadins, most of γ-gliadins and a few β-gliadins and the *Gli-2* loci on the short arm of homoeologous group-6 chromosomes for all of the α- and β-gliadins and γ-gliadins. The loci and chromosomes belong to the E or J genomes in the genus *Thinopyrum*, (from diploid to decaploid) and to the A, B and D genomes in the genus *Triticum* (diploid, tetraploid, hexaploid) (Payne et al. 1982; Dvorak et al., 1986). Glutenins are proteins separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) into high-molecular-weight glutenin subunits (HMW-GSs) and low-molecular-weight glutenin subunits (LMW-GSs) (Jackson et al. 1983). The *Glu-1 loci* encode for the HMW-GSs. Each *Glu-1* locus has two linked genes designated as x- and y-type based on differences in molecular weight, number of cysteine amino acids and repetitive motifs. LMW-GSs consist of subunits encoded by a multigene family, *Glu-3* loci. LMW-GSs are divided into B, C, and D groups according to the molecular weight and isoelectric point (Jackson et al. 1983). The *Glu-1* loci are located on long arm of homoeologous group 1 chromosomes and *Glu-3* on the short arm of the same homoeologous group 1 linked to *Gli-1* loci (Cassidy et al. 1998) of the E or J^e genomes in tall wheatgrass and A, B, D genomes in wheat.

In tall wheatgrass, as in wheat, gliadins and glutenins (HMW-GS and LMW-GS) are a source to identify novel polymorphism at all protein loci, and genotypes with quality associated protein components (Moustakas et al. 1988; Che et al. 2004; Cassidy et al. 1998; Feng et al. 2004; Liu et al. 2008 a, b; Luo et al. 2005; Wang et al. 2006). Relevant information, about the chromosomal location of encoding genes for gliadin and for a series of novel HMW glutenin subunits identified in *Thinopyrum* or *Agropyron* and data on their correlation with good bread-making quality, have long been published (Soliman et al. 1980; Dvorak et al. 1986; Luo et al. 2005; Wang et al. 2006; Liu et al. 2008b; Garg et al. 2009; Li et al. 2012).

Different studies also emphasized the potential value of novel germplasm in wheat breeding, reporting results on the production of hybrids, introgression and addition lines (Feng et al. 2004; Garg et al. 2009; Niu et al. 2011;), evolutionary process of genes and moreover on proteome response to severe water stress and recovery (Feng et al. 2004; Luo et al. 2005; Liu et al. 2008a; Liu S. et al. 2007; Gazanchian et al. 2007). At the present no data on seed storage proteins of *Thynopyron elongatum* collected in Apulian coastal wetland, have been published along with information on possible correlation with plant communities and ecological context.

In consideration of the complexity of the aspects linked to the monitoring of coastal wetland, some natural populations of *Thynopyron elongatum* and the surrounding plant communities were explored applying a multidisciplinary approach, in particular, to achieve the following purposes: (1) assessment of vegetazion zonation and ecological context of three coastal wetlands; (2) characterization of habitats and plant communities in which *Thinopyrum elongatum* occurs, by means of vegetation and soil samples analysis; (3) assessment of genetic diversity of seed storage proteins, gliadins and glutenins, extracted from seeds of the twenty-four populations of *Thinopyrum elongatum,* in order to study polymorphism and determine genotype frequencies and distribution in relation to the surveyed plant communities and habitat.

Vegetation zonation plant communities, ecological context of the three coastal wetlands, *Thinopyrum elongatum* genotypes frequencies and distribution, monitored over time, will be

indicators of biodiversity changes and would assist in the planning of future conservation actions.

Materials and Methods

Study area

Apulia (Puglia in Italian) forms the eastern most part of the Italian peninsula and is the Italian Administrative Region with the longest part of the coastline (900 km). This coastline consists of 38% beaches, 57% rocky coasts and 5% urban areas. Coastal wetlands characterize sandy shores and most of these are protected areas (Boenzi et al. 2006). According to Rivas Martinez et al. (2004), this region belongs to the Mediterranean pluviseasonal oceanic bioclimate, with thermotypes ranging between the thermo-Mediterranean and meso-Mediterranean, with dry to sub-humid ombrotypes, along with a general trend in aridity and a growing vulnerability to desertification (Blasi et al. 2007).

Three Sites of Community Importance (SCI), located along the coasts of Apulia and characterized by sandy shores and salt marshes were selected as study areas: 1) "Coastal dunes from Torre Canne to Torre San Leonardo "(SCI IT9140002 "Litorale Brindisino", 7255,855 ha); 2) "Punta della Contessa Salt Pans "(SCI IT940003 "Stagni e Saline di Punta della Contessa", 2858,158 ha); 3) "La Vela Swamp" (SCI IT9130004 "Mar Piccolo", 1347 ha). A high degree of human pressure characterizes the three areas and was the priority criterion choosen. The selected areas were indicated by the acronyms "S" for the Regional Natural Park "Punta della Contessa Salt Pans", "P" for Regional Natural Oriented Reserve "La Vela Swamp" and "T" for the Regional Natural Park "Coastal dunes of Torre Canne Torre San Leonardo."(Figure 1; Figure 2).

Vegetation sampling and analysis

Coastal vegetation of salt marshes was randomly sampled for two consecutive years during the summer (July- August). The Zürrich-Montpellier phytosociological method (Braun-Blanquet 1964) was applied to perform a total of 70 phytosociological relevés with plot size varying from of 200 m^2 to 50 m^2, depending on vegetation type and microtopography. Each relevé was georeferenced by GPS. A single matrix of 70 relevés x 56 species combined the different data collected. The Braun-Blanquet sampling scale was transformed into the ordinal scale according to Van der Maarel (1979). Similarity analysis of the relevés was carried out by using the SYN-TAX 2000 software (Podani 2001). A cluster analysis based on the Euclidean distance and the UPGMA linkage method was performed. Clusters were referred to plant communities (association) and the species frequency was calculated, in order to highlight the communities in which *Thinopyrum elongatum* is present and those where it appears more frequently.

In order to point out ecological patterns and to generate hypotheses about vegetation-environment relationships, an ordination of the data set was carried out by using the PC-ORD 6 software (McCune & Mefford, 1997). In the ordination analyses the Detrented Correspondence Analysis (DCA) was ran, achieving a consistent ordination diagram.

Nomenclature of the taxa cited in the text is in agreement with Bartolucci et al. (2018), except for *Thinopyrum elongatum* for which we refer to Wang (2011). Vegetation units (of higher-level respect to association) were assigned to the different syntaxa according to Biondi et al. (2014) and to Mucina et al. (2016). ***Soil sampling and analysis***

Seven soil samples, one for each plant group identified by the multivariate analysis, were performed and analysed in order to verify the ecological context suggested by field observation and by the ordination diagram. The pedological factors were described according to the FAO classification 2006 (IUSS Working Group WRB. 2007). The laboratory analyses were done following the Soil Surveyof the USDA (Soil Conservation Service USDA, 1972) and its updating. The factors analysed are: conductivity 25° (mS/cm), salinity (g/100 g), organic matter (%), soil texture (presence of clay, silt, sand in %).

Plant sampling

The plots of vegetation sampling (phytosociological relevés) were considered also as sites for the plant sampling and related seeds for biochemical analyses.

In each specific vegetation plot, one or more 2 m × 2 m plots, georeferenced by using Global Positioning System (GPS), were random detected for the collection of ears per plant, trying to cover all areas where *Thinopyrum elongatum* was present. Individual ears were cut off at their base and placed in paper bags. Date, place of collection, species, number of the sample and any other annotations on the characteristics of the collection point, were recorded. Each spike was signed by a capital letter corresponding to the origin area (S, P, and T) and by sequential number indicating the sequence of harvesting sites. After field collection, the samples were dried for a few days in a warm and dry camera, to allow the loss of the residual moisture. Manual cleaning of the kernels from the ears removing the adhering lemma and palea, was also performed.

About 100 ears were picked for biochemical analysis. The plant populations collected, were eight in the site S and eight respectively in the P and T. The sites were conventionally marked with the capital letter S, P, T followed by the number of each harvesting sites (from 1 to 8).

Biochemical analyses

One hundred ears for each population and collection site (S=8; P=8; TC=8) and from three to five kernels per ear were analysed. Each seed was treated as a single sample for electrophoretic analysis. Durum wheat Italian cultivar ‘Svevo’ and common wheat cultivar ‘Chinese Spring’ characterized by the presence of different allelic variants at gliadin and glutenin loci, were used as control. In addition to these two varieties, a marker of the molecular weight BenchMarkTM Protein Ladder (10-220 kDa), was also used.

The biochemical characterization, of storage proteins extracted from kernels of *Thinopyrum elongatum*, was performed by one- (A-PAGE, SDS-PAGE) and two-(IEFxSDS-PAGE) dimensional electrophoretic analysis.

Gliadins and glutenin components were extracted from wholemeal flour and separated in A-PAGE and SDS-PAGE (10% polyacrylamide gels) as described by Payne et al. (1982) and Margiotta et al. (1993). For glutenin, the samples were incubated with 100 µl Tris-HCl, 0,125M pH 6,8 extraction buffer containing 4% (w/v) SDS, 20% (v/v) glycerol, 1% DTT (w/v) and bromophenol blue.

After the run, A-PAGE and SDS-PAGE gels were stained overnight with 12% trichloroacetic acid solution containing 5% Comassie brillant blue R-250 in absolute ethanol (1%, w/v) and destained with distilled water for 12 hr.

Two-dimensional electrophoretic analysis (IEFxSDS-PAGE) of glutenin fractions, were performed following a modified procedure of Ikeda et al. (2006).

After the analyses, a lower letter was assigned to each electrophoretic profile or genotype identified.

Preparation of root tips for chromosome counting

Chromosome counting, of representative sample/genotype per each population was performed by the squash method (Rayburn A.L. and Gill B.S. 1985). Root tips, collected from germinated seeds in petri dishes, were pre-treated with ice water (0°C) for 24–28 h and fixed in 3:1 ethanol/acetic acid and finally squashed and stained in 2% acetic carmine. (*Thinopyrum elongatum*, 2n=14).

Gel acquisition, image analysis and graphical representation

All gels produced were acquired by the image acquisition system "Chemi Doc" and the Bio-Rad protein components in each electrophoretic profile highlighted and analyzed using the program "Quantity One" (Bio Rad). The image analysis allowed the detection of differences among electrophoretic patterns.

The frequencies (% representatives) of different genotypes identified in each site and area, both for gliadin and glutenin components, in particular for HMW-GS, were also determined. These were calculated for each site and area considering the number of identical electrophoretic patterns (genotypes) identified on the total of seeds analyzed. Genotypes identified for each site were considered as the independent variables (x-axis), the corresponding frequencies (%) the dependent variables (y-axis) (Figure 10).

Nomenclature and protein identification

The protein compositions were determined using different *T. aestivum* and *T. durum* cultivars. In particular, cultivars bread wheat Chinese Spring and durum wheat Svevo, were included as controls respectively for gliadin and high *M*r glutenin subunits.

The gliadin and glutenin patterns were processed by a computerized system and subsequent visual inspection. The study of individual classes of protein components allowed a preliminary assessment of the genetic diversity of populations collected. It was thus possible to associate particular protein electrophoretic patterns or specific genotypes detected with higher and lower frequency to the data collected for the plant communities. Each genotype was indicated by a lower letter representative of gliadin or glutenin profile (a, b, c, etc.….) and an apix capital letter referred to the site of origin (a^S, b^S, c^S, etc., a^P, b^P, c^P, etc., a^T, b^T, c^T, etc., see Figures 6, 7, 8, 9, 10).

Results

Multivariate analysis of vegetation data, synoptic scheme and frequency of Thinopyrum elongatum in plant communities

The cluster analysis identifies two main clusters, A and B (Figure 3), separating the chamaephytic halophilous vegetation characterized by succulent dwarf shrubs (A) from the helophytic, halophilous grasslands of salt marshes (B), respectively corresponding to the *Salicornietea fruticosae* class and to the *Juncetea maritimi* class reported in Table 1. Cluster A splits in two main sub-clusters, A1 and A2 separating halophilous from halo-nitrophilous shrub communities. Cluster A1 splits into two main sub-cluster, A11 and A12. A11 includes the strictly halophilous shrub communities usually growing in the inner parts of the salt marshes characterized by *Salicornia perennis* subsp. *alpini* (A111) and by *Arthrocaulon*

macrostachyum, (A112). Cluster A12 includes the halophilous, chamaephytic and hemicriptophytic communities characterized by the dominance of *Limbarda crithmoides* subsp. *longifolia* (A121) and *Artemisia caerulescens* (A122). Cluster A2 groups the halo-nitrophilous shrub communities usually located in the higher and external parts of the salt marshes (*Suaeda vera* and *Halimione portulacoides* communities).

Cluster B is divided in two sub-clusters, B1 (*Schoenus nigricans* community) and B2 (*Sporobolus pumilus* community). *Sporobolus pumilus* communities are usually located in the most peripheral parts of the salt marshes, at the boundaries with the dune belts (often in contact). The main clusters were classified according to phytosociological literature and the syntaxonomical scheme is reported in Table 2.

Detrented Correspondence Analysis (DCA) was used to identify ecological patterns in the scattergram and to generate hypotheses about vegetation-environment relationships. The edaphic gradients seem to be particularly important for the vegetation of the brackish areas.

The ordination graph (Figure 4) points out the seven groups or communities, corresponding to six more or less defined ecological contexts. In the upper part of the graph, the *Suaeda vera* group (5) clearly separates from the rest of relevés. In the down lower part of the graph, five groups are evenly distributed along the axis 1, quite clearly identifying the presence of one or more ecological gradients. Along this axis, from left to right, it is possible to identify the *Arthrocaulon macrostachyum* (1) and *Salicornia perennis* subsp. *alpini* (2) communities, then the *Limbarda crithmoides* (3) together with *Artemisia coerulescens* (4), *Schoenus nigricans* (6) and finally, the *Sporobolus pumilus* (7) communities. Axis 1 clearly identifies the main ecological gradient, considered referring to a specific edaphic gradient mainly related to salinity, soil texture and moisture. Plant communities of the salt marshes are spatially ordered because of their microecological characteristics, usually forming concentric belts around the water body, and based on ecological gradients linked to edaphic features such as salinity and soil texture and/or to the length of the flooding period (soil moisture) (Rogel et al. 2000; Molina et al. 2003; Álvarez-Rogel et al. 2007). Plant communities dominated by *Arthrocaulon* and *Salicornia* tend to occupy the innermost belts, close to the water body and subject to more or less prolonged flooding periods. *Limbarda crithmoides* plant communities, such as those characterized by *Artemisia coerulescens*, are located along the external and more elevated belts enduring limited flooding periods. *Suaeda vera* plant communities usually are located in the most peripheral areal, often characterized by a high level of organic matter in soils and only occasionally subject to flooding. At last, perennial grasslands with *Sporobolus pumilus* are located on the borders between salt marshes and dune belts (Frondoni & Iberite 2002; Tomaselli et al. 2011; Tomaselli & Sciandrello, 2017; Tomaselli et al. 2017).

The synoptic table (Table 1) highlights the presence of *Thinopyrum elongatum* in almost all the examined plant communities, with the exception of the *Sporobolus pumilus* communities (group 7). *Thinopyrum elongatum* shows its relevant presence in groups 3-4-5-6, with the highest frequencies in 3 and 4, corresponding to *Limbarda crithmoides* and *Artemisia coerulescens* plant communities, respectively (*Agropyro elongati-Inuletum crithmoidis* and *Limonio narbonensis-Artemisietum coerulescensis*).

Soil analysis

The edaphic data support the outcomes of the ordination analysis. Values of conductivity, salinity, organic matter and texture were processed in histograms (Figure 5 a and b) to highlight the trend of ecological gradients. Vegetation types were organized in sequence in the histogram according to their natural arrangement in natural environments, as described in the previous section.

Histogram 5a, shows very clearly how the soil salinity, with the highest values in *Arthrocaulon macrostachyum* communities tends progressively to decrease towards the peripheral vegetation types. Histogram 5b, shows a gradient linked to soil texture. Clay and silt, high in the internal lower parts of the salt marshes, tend to decrease in peripheral belts, especially towards the dune belt (seaside). *Sporobolus pumilus* vegetation, located in the peripheral part of the salt marshes, close to the dune belt, grows on soils with a very high sand component (98.5%).

Seed storage proteins: genetic diversity of gliadins and glutenins

The protein components, separated by gel electrophoresis and stained in the gel, were often slightly coloured; the amount with respect to the total protein content was probably low. Thin bands, in A-PAGE and SDS-PAGE analysis, were considered and treated as novel allelic variants, at gliadin and glutenin loci, to ensure the detection of all genotypes for each population, inside the sites and areas (Margiotta et al. 2013a; Margiotta et al. 2013b). The results of analyses were simultaneously subjected to visual inspection and computed (by software Quantity One-BioRad). Moreover, in order to point out the differences between the genotypes, gliadin variability was assessed within each subclass (α, β, γ, and ω), while for the glutenins, in particular HMW-GS, were considered the ranges of apparent molecular weights (MW, kDa).

In wheat, genetic analysis of the electrophoretic patterns, obtained from the A-PAGE separation of gliadins, showed that these are inherited in the form of group or blocks of components and different allelic variants for each block were detected (Metakowsky et al. 2006). For *Thinopyrum elongatum* such allelic variants have not been reported and only few studies describe their frequencies.

The electrophoretic separation in A-PAGE (Figure 6) shows the genotypes selected for sites and areas.

In the area S, the eight electrophoretic patterns (a^S, b^S, c^S, d^S, e^S, f^S, g^S, h^S), represent all the variability detected within the eight populations collected in the eight sites (one genotype for each population). Genotypes detected in general differed for few bands in particular in the slow moving omega gliadin region.

The genotypes detected in area P (eight collection sites), showed higher variability for the alfa, beta, gamma and omega gliadins. The omega gliadins in particular possessed different electrophoretic mobilities and resulted in numerous respect to those observed in areas S and T. Six genotypes (a^P, b^P, c^P, d^P, e^P, f^P) were representative of all populations collected (Figure 6, P).

In the area T, the total amount of omega gliadins were intermediate to those observed for the same components in the area S and P and fine differences among all classes including alfa, beta, gamma, were considered relevant to detect all the available polymorphism. The genotypes c^T and d^T, e^T and h^T collected in the site T7 and q^T, r^T, in T8 (Fig. 6), resulted in similar pairs as gliadin components but were therefore considered different after the analyses of glutenin fraction. In fact, in the LMW-GS fraction, different protein components were detected (arrowheads in Figure 8).

Figures, 7 and 8 illustrate the SDS-PAGE separations of total proteins extracted from the same genotypes of *Thinopyrum elongatum* reported in Figure 6 and collected in the area S, P and T, and the polymorphism detected at glutenin loci in all sites. The classes of protein components considered were the HMW-GSs, the LMW-GSs and the ω gliadins.

The genotypes had all x- and y-type HMW-GSs with apparent molecular weights significantly different from those observed in cultivated wheats. In particular, the x-types were detected at molecular weights below the Dx of hexaploid wheats and the Bx of hexaploid and tetraploid used as standard (Sv and CS in Figures 7 areas S and P, and Figure 8 area T). These x type were associated with subunits of lower intensity, probably secondary products of degradation or with subunits of greater intensity falling within the interval of the molecular weights of the D type LMW-GS present in bread wheat. The range of molecular weights for the x-types varied from about 90 kDa to 100 kDa and for y from 60 to 90 kDa (Figure 7 standard of molecular weight adopted).

Similarities in terms of HMW-GS were detected in P area among genotypes a^P, c^P, d^P, f^P and between b^P and e^P and in the T area among all genotypes.

The number of genotypes in the area S, P and T corresponded to those selected for gliadin components, with one mention for the T area, regarding the class of LMW-GSs. In fact, as reported in Figure 6, inside two sites of this area T were detected three pairs of genotypes, the c^T and d^T (asterisks), e^T and h^T (empty circles) in the site T7 and q^T, r^T (black filled circles) in T8, previously described. They possessed the same gliadins and HMW-GSs but differed from each other for some LMW-GSs belonging to the B group of subunits (arrowheads in Figure 8).

Finally, the SDS-PAGE electrophoretic patterns, reported in Figures 7 and 8, which correspond to patterns reported in Figure 6, were considered representative of all polymorphism detected at gliadin and glutenin loci in the three areas.

The biochemical characterization of glutenin fraction was completed by two-dimensional electrophoretic separation. In particular, the glutenin fractions extracted from some representative genotypes b^P, d^P, e^T and b^S of *Thinopyrum elongatum* were compared to those of hexaploid wheat cultivar Chinese Spring and tetraploid wheat variety Svevo. The analyses of electrophoretic patterns revealed that the differences are mainly due to the acidic and basic behaviour of the protein components. The HMW-GS and LMW-GS and in general, the whole glutenin fractions of *Thinopyrum elongatum* extracted from the three areas, in particular the group of HMW-GSs, appeared more basic (Figure 9, bP, dP, eT and bS) than the corresponding classes present in tetra- and hexaploid wheat (Figure 9, CS and Sv).

Genotype frequencies

The histograms in Figure 10 represent the frequencies (percentage) of all genotypes detected in each site for each area. The histogram reports, in the abscissa axis, different sites explored or populations collected in the three areas S, P and T (S1, etc…, P1, etc., T1, etc…) and the ordinate axis the corresponding frequencies of each genotype (percentage). All genotypes for each area and site represent all variability detected for gliadin or glutenin components at gliadin and glutenin loci. The genotypes were grouped according to the differences noticed among gliadin (Figure 6) and glutenin (Figures 7 and 8) electrophoretic patterns. The same genotypes, with different frequencies, were identified in more than one site inside the same area.

The genotype b^S was the most frequent (33,3%) in the S area as it was present in five sites (S2, S5, S6, S7, S8) along with the d^S (20,83%) and the c^S (16,67%) in three different sites. Among the six genotypes of the area P, the most representative were the b^P and c^P (26,2% and 23,75%) present in P1, P3, P4 and P3, P4, P5 sites, while a^P and d^P, e^P and f^P were equally distributed in the site P2, P6, P7, P8. The genotypes detected in the area T were more numerous with respect to those observed in other areas S and P. In this area the most

representatives were the b^T and e^T (18,30%) and decreasing in percent the l^T, i^T, g^T as reported in the Figure 10.

Discussion and Conclusion

The activities of exploration and plant collection in the selected coastal wetlands allowed: a) the characterization of plant communities through vegetation survey, focusing on those hosting *Thinopyrum elongatum*; b) the monitoring of presence and distribution of *Thinopyrum elongatum* in the study sites; c) the analyses of polymorphism of seed storage proteins in the populations collected with the identification of the most representative genotypes, and d) the monitoring of each genotype of *Thinopyrum elongatum*, inside plant community.

Thinopyrum elongatum populations showed a significative presence in the halophile shrub plant communities of salt marshes, especially those characterized by *Limbarda crithmoides* and *Artemisia coerulescens,* corresponding to the two associations *Agropyro elongati-Inuletum crithmoidis* and *Limonio narbonensis-Artemisietum coerulescensis*, both belonging to the *Inulion crithmoidis* alliance. According to literature data, these plant communities are generally located in the most elevated and peripheral parts of the salt marshes, subject to sporadic and short flooding period along the year and usually characterized by moderate soil salinity, slight presence of organic matter and sand-silty soils. Our field observation, in addition to vegetation and soil analyses have confirmed the presence of such environmental conditions and have allowed the outlining of specific ecological gradients, linked to particular vegetation zonation, in the three sites. The association of *Thinopyrum elongatum* to these particular conditions (sandy or silty-sandy soils, moderate organic matter and salinity in particular hyposalic-salic soils) appears as a constant character in the three sites, even if the specific plant associations (communities) can be slightly different. In any case, the definition of a more precise ecological outline for *Thinopyrum elongatum* requires more detailed and extensive analyses, both in field and laboratory tests.

Thirty-four genotypes were identified in the analyzed populations: eight in S, six in P, and a much higher number, 20 in T. This higher number of genotypes in T, and in particular the differences of the protein patterns, suggest the presence of a higher genetic variability in T with respect to S and P areas. Such higher genetic variability could be related with the presence of a larger and better-conserved population of *Thinopyrum elongatum* in this site. It is well known that population genotypic diversity may have important positive ecological effects at the population, community (e.g. community structure) and ecosystem (e.g. ecosystem processes) levels, and in some cases such effects are comparable to those of species diversity (Cutsinger et al. 2006; Hughes et al. 2008). Genetic diversity is an important driver of ecological processes especially in communities or ecosystems dominated by one or a few primary habitat-providing species (Whitham et al. 2006; Hughes et al 2008). These effects are usually more evident in those habitats characterized by one or more dominant plant species (e.g., the case of *Thinopyrum junceum* or *Calamagrostis arenaria subsp. arundinacea* of embryonic or white dunes). In any case, a higher genetic diversity provides greater resistance of the community in case of anthropic disturbance. The populations of *Thinopyrum elongatum* observed and sampled in the site T are the more extensive of the three study sites. Their pronounced genetic variability lets us to predict a greater resistance to the numerous factors of human pressure affecting the three sites inside the coastal areas considered in this study.

Focusing on the specific genotypes, the b^S, b^P, b^T were the most frequent in the three sites S, P and in T, even though inside the areas S and P respectively genotypes d^S and c^P were also common. The differences among all genotypes were essentially due to the differences in alfa, beta and gamma gliadin composition for genotypes belonging to the areas S and T, while in P essentially in omega gliadin composition. In the case of P seems that it has been favoured by

the accumulation of low-sulfur proteins, such as the omega-gliadins whose major presence in percent respect to alfa, beta, gamma gliadins is generally associated to sulfur deficiency during seed development and germination. The genotypes in T also showed in particular that these differences in alfa, beta and gamma gliadins were consequently associated to the differences in LMW-GSs while, on the contrary they presented the same HMW-GSs. Moreover, it was observed that in general the types of protein components belonging to each class of gliadins and glutenins seem to possess mainly basic biochemical properties confirming the adaptation and the survival of the plant to environment with very saline soils.

Regarding the correlation between the identified genotypes and the collection sites (or plant communities), genotypes c^P and d^P, among the most frequent types in Palude la Vela, were collected in *Limbarda crithmoides* communities (*Agropyro elongati-Inuletum crithmoidis*) as well as c^T and d^T, in Torre Canne, on slightly salty soils. Genotypes b^S, b^P and b^T, the most frequent in all sites, even do not seem to be related to a particular plant community, were found in different plant communities in the three sites showing the highest diffusion and the highest capacity of adaptation. Genotypes c^S and d^S, among the most frequent in the site Saline di Punta della Contessa, were collected in *Artemisia coerulescens* communities, that is the *Limonio narbonensis-Artemisietum coerulescentis* association. Genotypes e^T, l^T seem to be related to the *Salicornia perennis* communities (*Puccinellio-Sarcocornietum alpini*) in the Torre Canne site, on soils with higher salinity and sand component, implying a generally more xeric environment.

Conservation of the environment often means “ex situ” and in particular “in situ” conservation of species and plant communities and a guarantee for further evolutionary adaptation (Hammer et al. 2003). Changes in genetic composition of plant communities, in particular habitat or environmental conditions, may influence and modify the species composition and structure of plant communities and often these modifications can be predicted studying the genotypic diversity of their component populations (Booth & Grime 2003; Whitlock et al. 2007). The identification of a correlation between particular genotypes or specific composition/genetic structure of plant communities, under specific environmental conditions, although requires more extensive investigation and in-depth analysis, already shows the effectiveness of the multidisciplinary approach adopted ~~applied in this study~~ and the adequacy of all experimental designA greater number of sites in other areas of the Apulia and, in general, in the Mediterranean area with similar characteristic should be taken into to follow biodiversity changes of habitats in wetland areas. A long term monitoring of vegetation zonation, plant communities, ecological context and genotypes of *Thinopyrum elongatum*, repeated over time, could become a practical system to control the anthropogenic pressure and consequently define plans for biodiversity conservation in the wetland protected areas safeguarding tertiary gene pools also valuable for future wheat genetic improvement.

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Disclosure statement

The authors disclose any conflict of interest.

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Table 1. Synoptic table with the frequencies (in percentage) scored by species for each analysed plant community.

DIAGNOSTIC SPECIES							
Plant communities	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Arthrocaulon macrostachyum	100,0	53,8	28,6	28,6	16,7	16,7	.
Salicornia perennis subsp. alpini	46,2	100,0	64,3	.	.	33,3	.
Thinopyrum elongatum	7,7	23,1	85,7	100,0	66,7	66,7	.
Limbarda crithmoides subsp. longifolia	23,1	38,5	100,0	100,0	.	100,0	18,2
Limonium narbonense	46,2	92,3	92,9	100,0	100,0	100,0	9,1
Juncus subulatus	76,9	61,5	7,1	57,1	33,3	.	9,1
Puccinellia festuciformis subsp. festuciformis.	53,8	100,0	14,3	85,7	.	.	.
Artemisia caerulescens	.	.	.	100,0	.	.	.
Suaeda vera	.	.	.	.	100,0	.	.
Schoenus nigricans	.	.	.	.	.	100,0	27,3
Plantago crassifolia	.	.	.	.	.	100,0	9,1
Sporobolus pumilus	.	.	.	.	.	.	100,0
Sarcocornietea fruticosae class							
Halimione portulacoides	92,3	100,0	78,6	100,0	100,0	50,0	.
Triglochin barrelieri	15,4	23,1	.	.	.	.	.
Aeluropus littoralis subsp. littoralis	15,4	.	35,7	.	.	.	.
Thinopyrum acutum	.	.	42,9	57,1	100,0	33,3	27,3
Limonium virgatum	.	.	21,4	.	.	83,3	9,1
Juncetea maritimi class							
Juncus acutus subsp. acutus	15,4	46,2	85,7	57,1	.	100,0	90,9
Juncus maritimus	23,1	30,8	100,0	.	.	83,3	72,7
Galatella pannonica subsp. pannonica	.	23,1	42,9	14,3	.	33,3	.
Carex extensa	.	.	7,1	.	.	50,0	9,1
Scirpoides holoschoenus	.	.	.	.	.	16,7	9,1

Table 2. Syntaxonomical scheme

SALICORNIETEA FRUTICOSAE Br.-Bl. et Tx. ex A. Bolòs y Vayreda et O. de Bolòs in A. Bolòs y Vayreda 1950
SALICORNIETALIA FRUTICOSAE Br.-Bl. 1933
Arthrocnemion glauci Rivas-Mart. et Costa M. 1984
Arthrocnemo-Juncetum subulati Brullo & Furnari 1976
Puccinellio festuciformis-Sarcocornietum alpini Castroviejo & Cirujano 1980
Inulion crithmoidis Brullo & Furnari 1988
Agropyro elongati-Inuletum crithmoidis Br.-Bl. (1931)1952
Limonio-Artemisietum coerulescentis (Pign. 1953) Géhu et Scopp. 1984
Suaedion brevifoliae Br.-Bl. et O. de Bolòs 1958
Halimiono-Suaedetum verae Molinier &Tallon1970 corr. Géhu 1984
JUNCETEA MARITIMI Br.-Bl. in Br.-Bl., Roussine &Nègre 1952
JUNCETALIA MARITIMI Br.Bl. ex Horvatic 1934
Juncion maritime Br.Bl. ex Horvatic 1934
Spartino-Juncetum maritimi O. Bolòs 1962
Plantaginion crassifoliae Br.-Bl. in Br.-Bl., Roussine &Nègre 1952
Schoeno nigricantis-Plantaginetum crassifoliae Br.-Bl. in Br.-Bl., Roussine &Nègre 1952

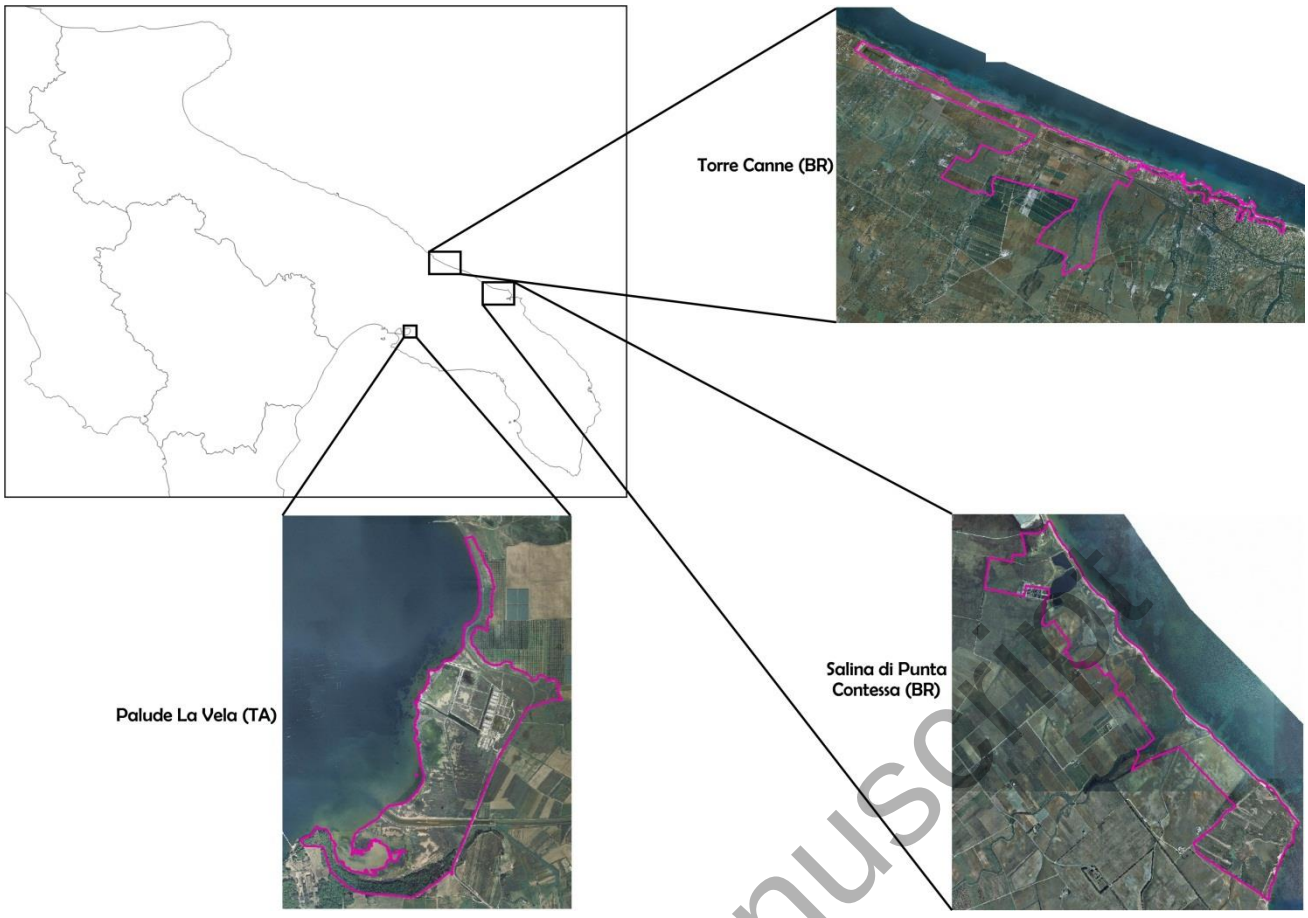

Figure 1. The three study sites and their location along the coasts of the Apulia region. In red, the SCI (Sites of Community Importance) boundaries.

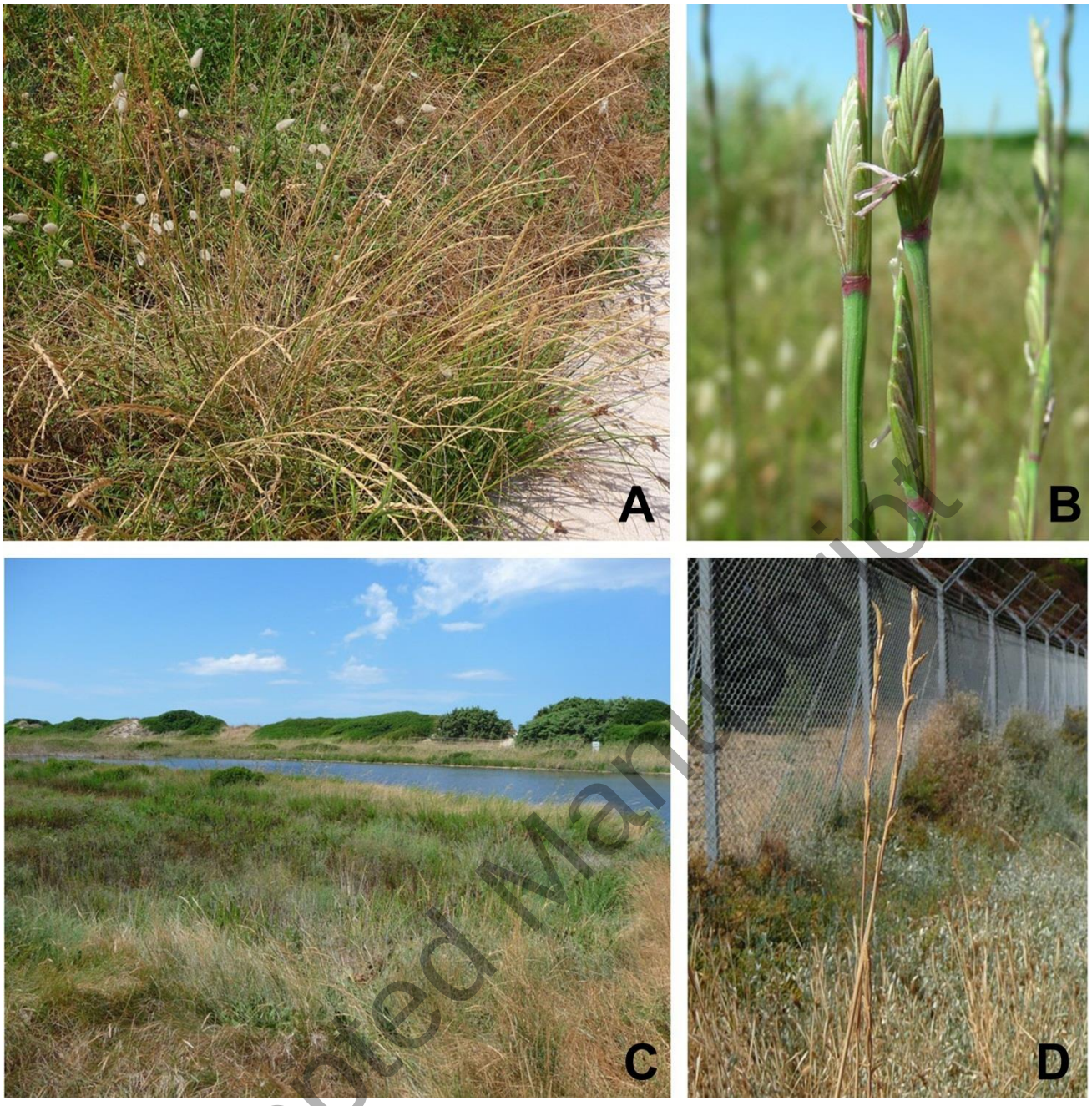

Figure 2. Regional Natural Park "Coastal dunes of Torre Canne Torre San Leonardo" (A, B, C) and Regional Natural Oriented Reserve "La Vela Swamp" (D). In B is reported a particular of *Thinopyrum elongatum*.

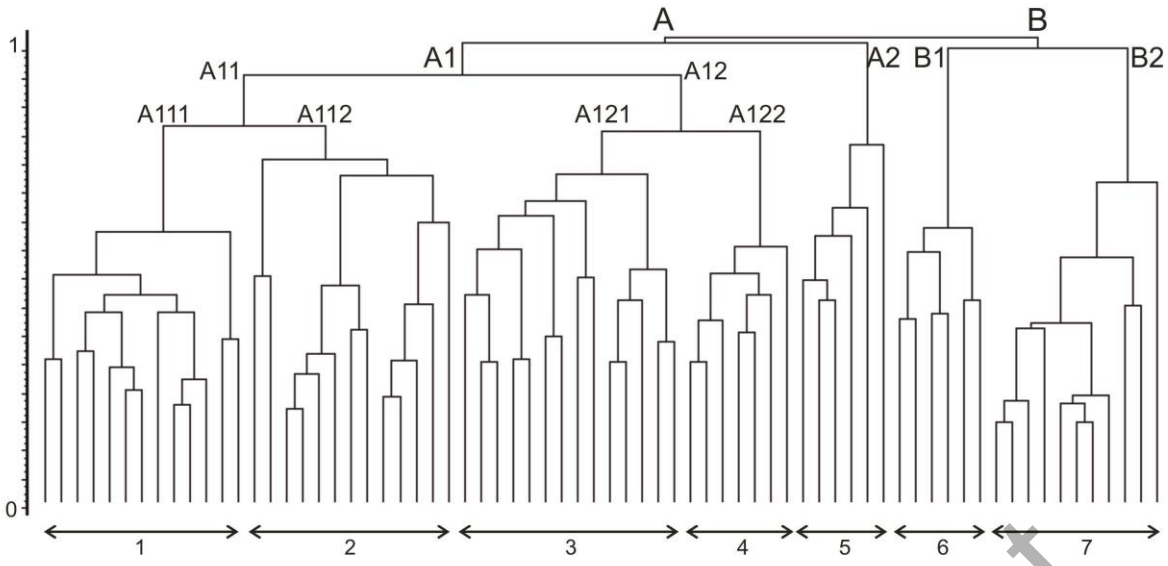

Figure 3. Dendrogram from the cluster analysis (Euclidean distance, UPGMA) of the data-set: 1 – *Arthrocaulon macrostachyum* communities (*Arthrocnemo-Juncetum subulati*); 2 – *Salicornia perennis* subsp. *alpini* communities (*Puccinellio festuciformis-Sarcocornietum alpini*); 3 – *Limbarda crithmoides* subsp. *longifolia* communities (*Agropyro elongati-Inuletum crithmoidis*); 4 - *Artemisia caerulescens* communities (*Limonio narbonensis-Artemisietum coerulescentis*); 5 - *Suaeda vera* communities (*Halimiono-Suaedetum verae*); 6 – *Schoenus nigricans* communities (*Schoeno nigricantis-Plantaginetum crassifoliae*); 7 – *Sporobolus pumilus* communities (*Spartino-Juncetum maritimi*).

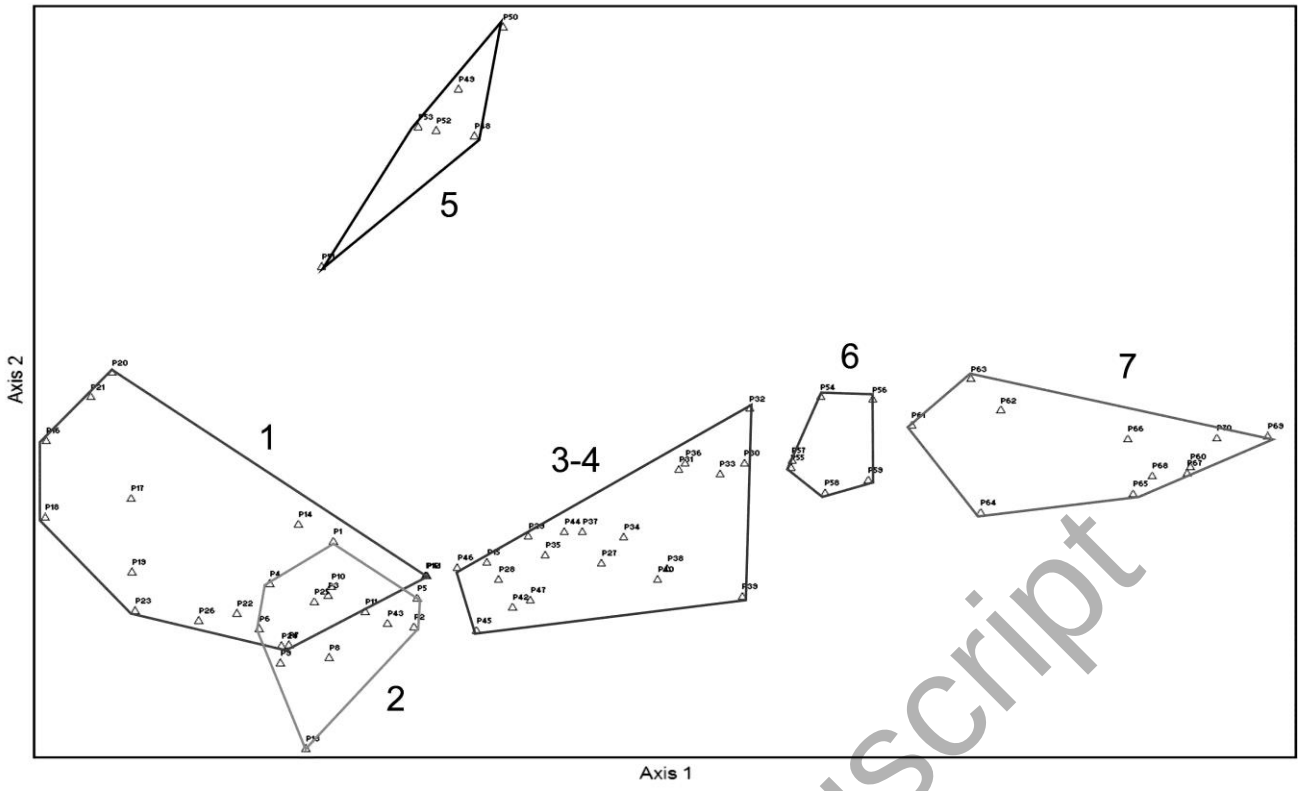

Figure 4. DCA ordination of the data-set, 2D (axis 1-2): 1 – *Arthrocaulon macrostachyum* communities (*Arthrocnemo-Juncetum subulati*); 2 – *Salicornia perennis* subsp. *alpini* communities (*Puccinellio festuciformis-Sarcocornietum alpini*); 3 – *Limbarda crithmoides* subsp. *longifolia* communities (*Agropyro elongati-Inuletum crithmoidis*); 4 - *Artemisia caerulescens* communities (*Limonio narbonensis-Artemisietum coerulescentis*); 5 - *Suaeda vera* communities (*Halimiono-Suaedetum verae*); 6 – *Schoenus nigricans* communities (*Schoeno nigricantis-Plantaginetum crassifoliae*); 7 – *Sporobolus pumilus* communities (*Spartino-Juncetum maritimi*).

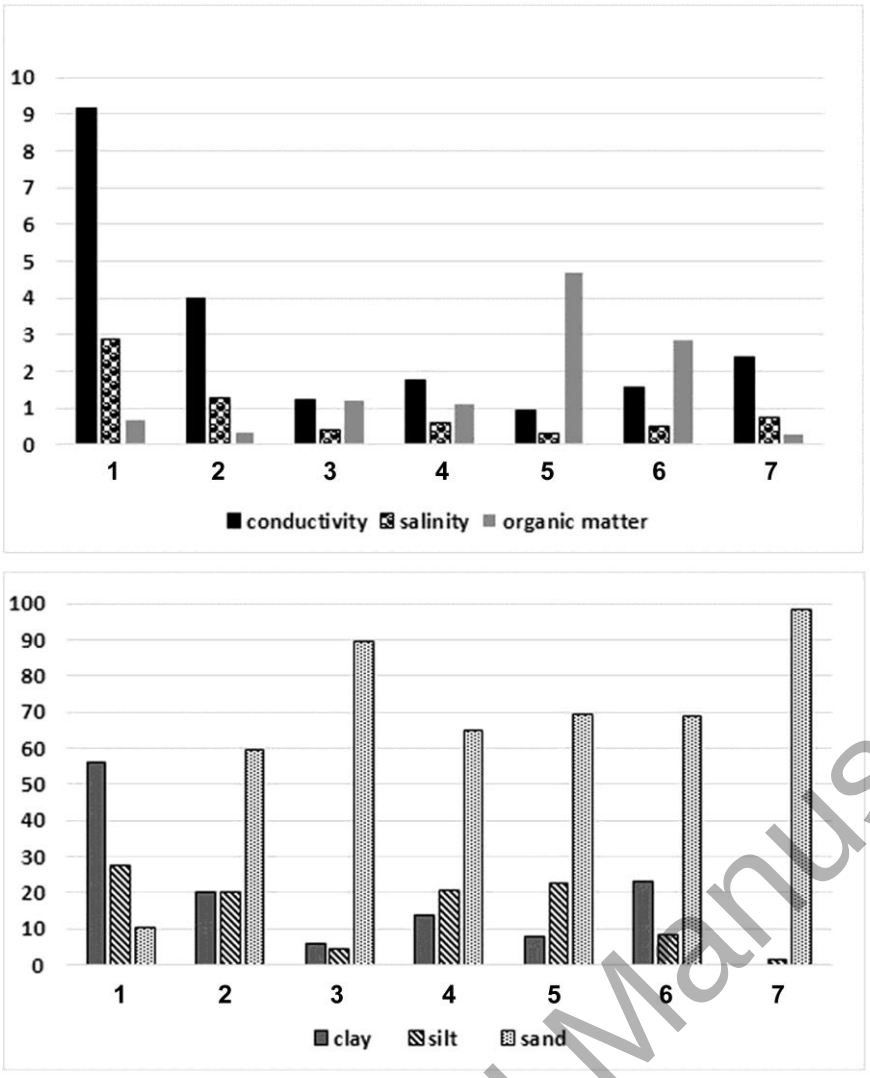

Figure 5. Histogram (a) showing conductivity, salinity, organic matter of the surveyed vegetation types and histogram (b) showing soil texture of the surveyed vegetation types: 1 – *Arthrocaulon macrostachyum* communities (*Arthrocnemo-Juncetum subulati*); 2 – *Salicornia perennis* subsp. *alpini* communities (*Puccinellio festuciformis-Sarcocornietum alpini*); 3 – *Limbarda crithmoides* subsp. *longifolia* communities (*Agropyro elongati-Inuletum crithmoidis*); 4 - *Artemisia caerulescens* communities (*Limonio narbonensis-Artemisietum coerulescentis*); 5 - *Suaeda vera* communities (*Halimiono-Suaedetum verae*); 6 – *Schoenus nigricans* communities (*Schoeno nigricantis-Plantaginetum crassifoliae*); 7 – *Sporobolus pumilus* communities (*Spartino-Juncetum maritimi*).

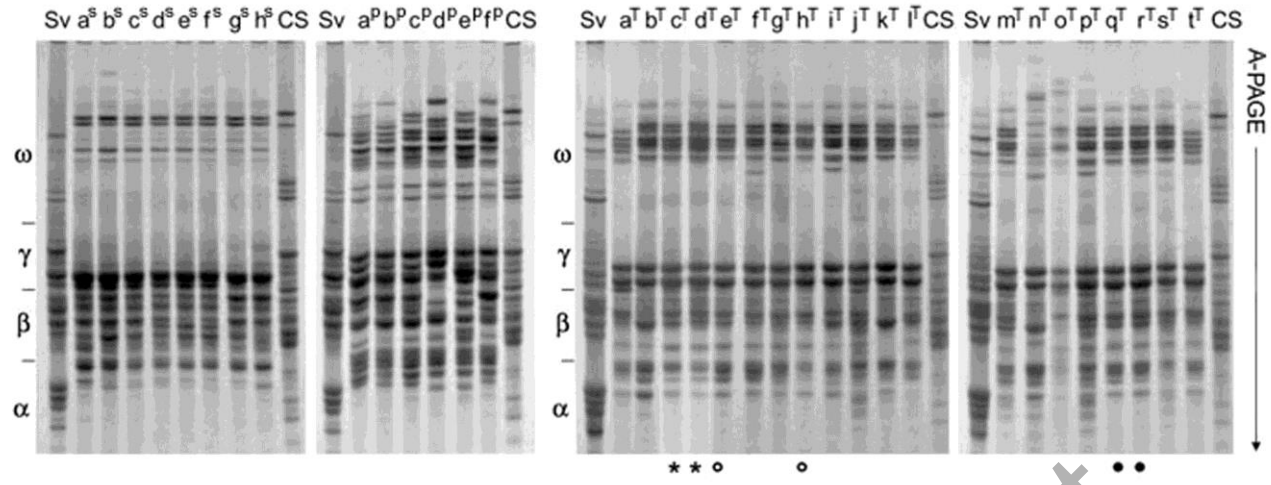

Figure 6. One-dimensional electrophoretic separations (A-PAGE) of gliadins from seeds of *Thinopyrum elongatum* collected in the area S, P, T and durum and bread wheat cultivar Svevo (Sv) and Chinese Spring (CS) used as standards.

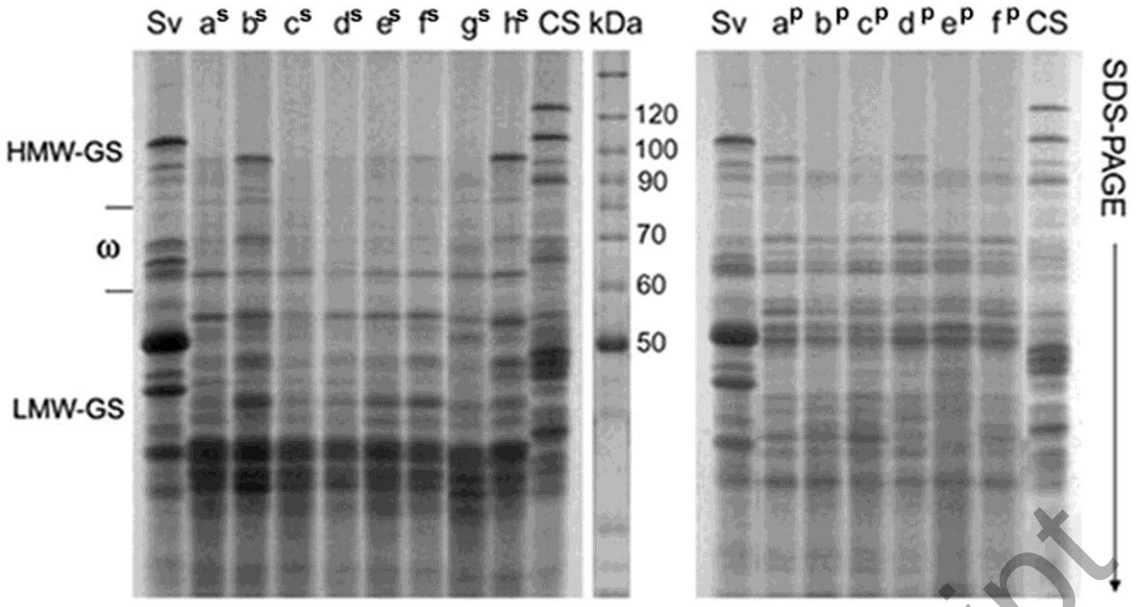

Figure 7. One-dimensional electrophoretic separations (SDS-PAGE) of total proteins extracted from seeds of *Thinopyrum elongatum* collected in the area S (a) and P (b).

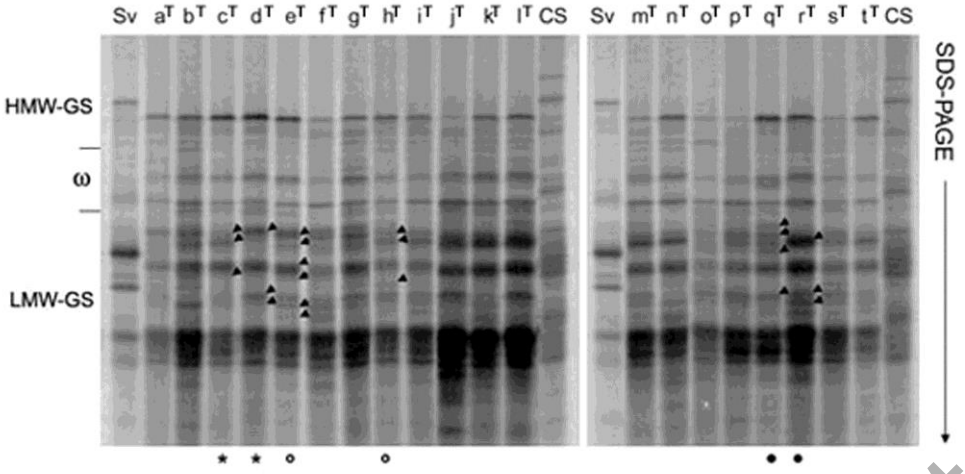

Figure 8. SDS-PAGE separations of total proteins extracted from seeds of *Thinopyrum elongatum* collected in the area T. The presences of different pairs of genotypes (asterisks, empty circles, black filled circles) and types of LMW-GSs (arrowheads), were indicated (see Figure 6 and comment in the text).

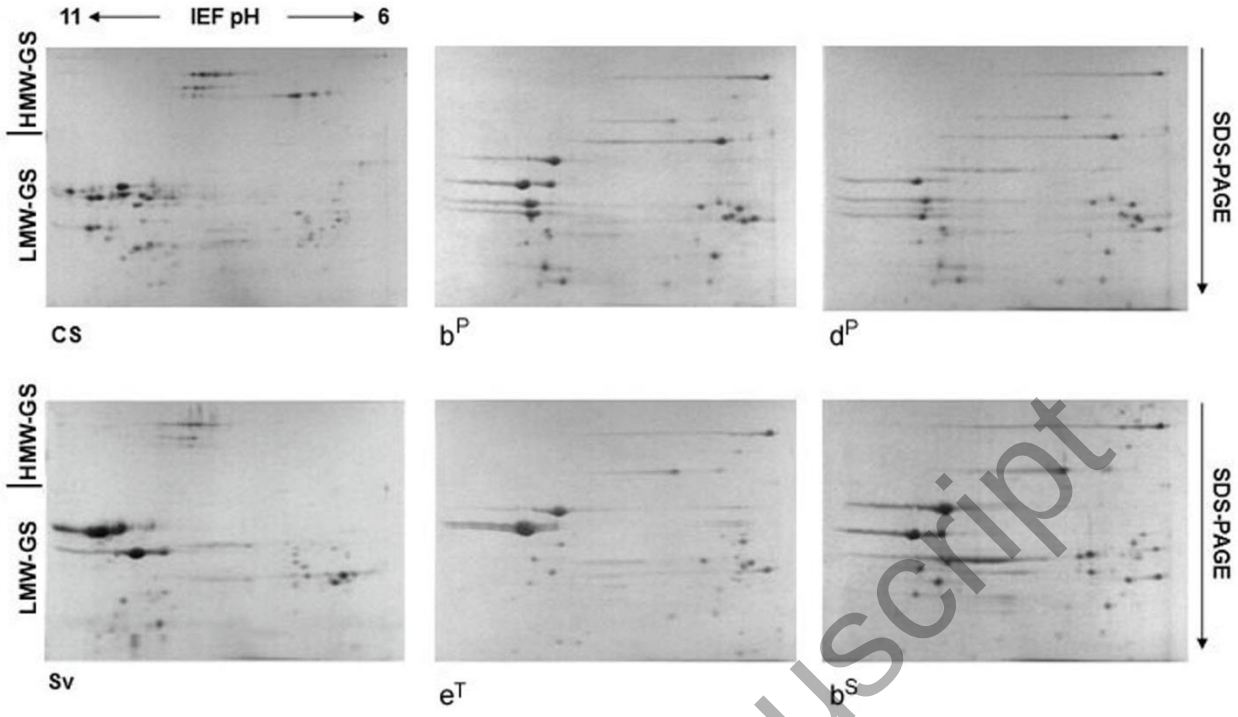

Figure 9. IEFxSDS-PAGE separations of glutenin fraction from hexaploid wheat cv. Chinese Spring (CS), tetraploid wheat variety Svevo (Sv) and four genotypes (b^P, d^P, e^T and b^S) of *Thinopyrum elongatum* collected in P, T, and S sites.

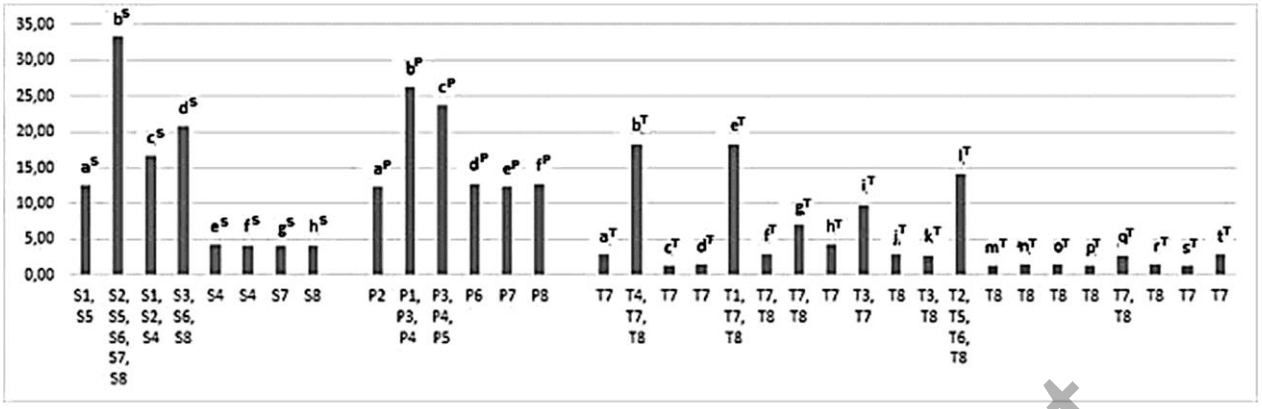

Figure 10. Frequencies (%) of genotypes identified for gliadin and glutenin components in sites (from 1 to 8) of the areas S, P and T.