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Genetic and morphologic variability of annual glassworts (*Salicornia* L.) from the Gulf of Trieste (Northern Adriatic)

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Abstract

The genetic variability of four pre-determined morphotypes of *Salicornia* (*S. patula*, *S. emerici*, *S. veneta* and the ''saline type'') from 10 locations on the Gulf of Trieste coast were studied by means of ploidy level estimation using flow cytometry and by molecular DNA analysis of ITS regions of nrDNA and cpDNA. Two groups, the diploids and tetraploids, with matching nrDNA sequences, were recognized. Two types of cpDNA emerged among the diploids; one the same as in tetraploids. This incongruence between nrDNA and cpDNA sequences indicates a hybridization with tetraploid maternal progenitors and demonstrates the evidence for reticulate evolution. The morphometry, based on generative morphological traits, did not clearly separate the four morphotypes. However, the most important characters—length of the middle fertile segment, length of the lateral flower, width of the scarious margin of the fertile segment in the floral region, conform to two genetically recognized types: diploid *S. patula* and the widely distributed tetraploid *S. emerici*, also comprising the ''saline type'' and morphotype, known as a charismatic endemic *S. veneta*, a flagship species for nature conservation. Other discriminative traits for diploid and tetraploid morphotypes are parameters of the flowers (comparison of length of the central vs. lateral flower) and stomatal index. The determination key is also given. The tetraploid *S. emerici* is by far the most common species of annual glassworts in the area, occupying more extreme habitats than a diploid *S. patula*, which mostly forms monodominate stands.

Keywords: *Salicornia emerici*; *Salicornia veneta*; *Salicornia patula*; Gulf of Trieste; cpDNA; nrDNA; Flow cytometry; Morphologic variability

1. Introduction

The annual glassworts (*Salicornia* L., Chenopodiaceae) are halophytic herbs with articulated succulent stems (Davy et al., 2001). Their extreme phenotypic plasticity (Ingrouille and Pearson, 1987), related to salinity–moisture–nutrients gradients, inbreeding and reduced leaf and flower morphology, provides few taxonomical characters (Kadereit et al., 2007). Dried herbarium material has very weak significance in comparison with fresh succulent plants (Davy et al., 2001; Kadereit et al., 2006, 2007), and the best way to unify sampling procedures is to exclude it. Dealing with fresh material only often means narrowing the research area or prolonging the sampling period over many years.

Although numerous species aggregates, species and microspecies have been described over the last 250 years in attempts to represent the variation observed, there is still no satisfactory taxonomic treatment, and it is frequently impossible to assign published information specifically to taxa within *Salicornia* (Davy et al., 2001). On the other hand, Ball (1993) in *Flora Europaea* and Stace (1997) recognize only three species: *S. pusilla* J. Woods, *S. europaea* L. aggr. (incl. *S. ramosissima* J. Woods, *S. europaea* L. and *S. obscura* P.W. Ball & Tutin) and *S. procumbens* Smith aggr. (incl. *S. nitens* P.W. Ball & Tutin, *S. fragilis* P.W. Ball & Tutin and *S. dolichostachya* Moss). One-flowered cymes of *S. pusilla* make it easily distinguishable from other three-flowered species. Aggregate *S. europaea* is associated with the section Salicornia sensu Scott (1977) and represents a diploid group ($2n = 18$) that is very variable because of autogamy. Aggregate *S. procumbens* is associated with the section Dolychostachiae of Scott (1977) and represents the tetraploid group ($2n = 36$).

Many morphological studies, aiming to delineate taxa and populations on the basis of morphometric studies were

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performed in Europe. One of the earliest was published by Ball and Tutin (1959) from Britain and Langlois (1961) from France. Ball (1964) published the taxonomic revision of *Salicornia* from Europe. Further studies included also ecological factors (Ball and Brown, 1970; Wilkon-Michalska, 1985; Ungar, 1987). More recent studies have applied numerical methods to morphometric data (Huiskes et al., 1985; Ingrouille and Pearson, 1987; Ingrouille et al., 1990). One of the most comprehensive data about taxonomic classification with determination keys is collected in Lahondère (2004). Early work was either contradictory (Noble et al., 1992) or had a crude taxonomic resolution (i.e. genus level; Luque et al., 1995).

The phylogeny of the Salicornioideae group worldwide (comprising of 14–16 genera and cca. 90 species), based on ITS nrDNA sequences and *atpB–rbcL* spacer (Kadereit et al., 2006), shows the annual and perennial glassworts (*Salicornia*/*Sarcocornia*) as one lineage (complex). A narrower phylogenetic analysis again based on ITS nrDNA, shows that the annual genus *Salicornia* is a sister group of the perennial genera *Sarcocornia*, *Arthrocnemum* and *Halocnemum* (Papini et al., 2004). Within four species of *Salicornia* only two groups were distinguished, which corresponded well with the ploidy level—one with the diploids, another with the tetraploids (Papini et al., 2004).

The aim of this present work is to elucidate the taxonomic background of the morphologic variability of annual glassworts, predominately by cytological and molecular means within a relatively narrow area, the coast of the Gulf of Trieste, which contains a wide range and a high proportion of very suitable habitats for *Salicornia*.

Many different interpretations of the species within *Salicornia* have been used for both the Slovenian and the Italian coasts, e.g. *S. europaea*, *S. ramosissima* Woods, *S. patula* Duval-Jouve, *S. emerici* Duval-Jouve and *S. veneta* Pign. et Lausi are mentioned in different combinations (Lausi, 1969; Pignatti, 1982; Iberite, 1996, 2004; Poldini, 2002; Jogan, 2007).

It was therefore necessary to seek the help of cytogenetic (ploidy level estimation) and molecular analysis, of both nrDNA and cpDNA, especially because of the high level of phenotypic noise and the scarcity of real diagnostic features serving to validate or reject the pre-determined morphotypes. We followed these steps: (a) sampling of apparently typical plants of each morphotype in their characteristic habitats; (b) measurement of morphologic traits; (c) determination key; (d) determination of the relative genome size and ploidy level via flow cytometry; (e) determination of the ITS nrDNA sequences from selected exemplars; (f) determination of the cpDNA sequences from the same exemplars; (g) on the basis of matching the ploidy, cpDNA, nrDNA, morphology and habitat requirements/preferences, proposal of the most suitable taxonomic scheme.

2. Methods

2.1. Study area and Salicornia morphotypes

The study area was the Gulf of Trieste, which includes the complete Slovenian coast from the Croatian border on the Savudrija peninsula to the Laguna di Grado on the Italian side of the border. Localities with appropriate habitats for glassworts were chosen: (1) the Sečovlje (45°28′N, 13°37′E) salt pans; (2) the Strunjan (45°32′N, 13°36′E) salt pans; (3) the Nature reserve of Škocjan inlet (45°33′N, 13°45′E) near Koper; (4) the botanical reserve of Sv. Katarina (Ankaran; 45°34′N, 13°44′E); (5) shell deposits near Ankaran; (6) Laguna di Grado (45°43′N, 13°26′E)–Primero: the lagoon; (7) Laguna di Grado–Primero: the fishing port; (8) Laguna di Grado: Lido di Stranzano; (9) the Nature reserve Lisert (45°46′N, 13°31′E) near Monfalcone; (10) the coast at Lisert. All the localities consist of muddy alluvial deposits of natural or anthropogenic origin (e.g. the Lisert mud deposits or the salt pans at Sečovlje and Strunjan), with high levels of salinity. The salt pan localities are closed and not exposed to a tidal regime.

According to observed ecological and morphometric variability of glassworts in the field, the following four morphotypes were recognized: type 1, the ‘‘saline type’’ (cylindrical, medium-sized fertile segments); type 2, the type from closed coasts (typical *S. emerici*—cylindrical, large fertile segments); type 3, the pyramidal prostrate type from open coasts (typical *S. veneta*—tall, pyramidal habitus, cylindrical medium-sized fertile segments); type 4, the small-flowered type from dry habitats (*S. patula*—inflated, small, short fertile segments). Type 1 occurs on the closed salt pans of Sečovlje and Strunjan Saline—with very limited access to fresh-water flow. Type 2 is the most widespread and inhabits wet mudflats, especially on the closed coasts. Type 3 is characteristic of open coast of almost tabular mudflats in the Laguna di Grado, but also occurs on other open coasts (e.g. near Ankaran, Slovenia). Type 4 is rare and appears on drier (high elevation) conditions in muddy or sandy soil, away from tidal oscillations.

2.2. Sampling procedures

Sampling was based on the four preliminarily determined morphotypes. Plants for the genetic analyses were collected in the localities listed in the study area. Only representative specimens of each morphotype were sampled.

For the preliminary nuclear DNA ITS analysis, samples were collected in September 2005. Additional sampling was performed in October 2006 to determine the nuclear DNA content: 14 samples representing various locations and morphotypes were used in the analysis.

For the morphologic analyses, eight fully grown plants were sampled on typical habitats from the six localities among previously mentioned: (1)–(5), and (7) in October 2006.

2.3. Analysis of nuclear DNA content by flow cytometry

Stem segments were collected from mature plants. The total DNA amount in the stem tissue nuclei was assessed by flow cytometry, using either propidium iodide, according to Doležel et al. (1989), or DAPI, according to Bohanec (2003). Several internal standards were tested (*Petunia hybrida*, *Lycopersicum esculentum*, *Trifolium pratense*, *T. repens*) as appropriate. Finally we used only the staining procedure based on DAPI