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Phenotypic traits of *Phragmites australis* clones are not related to ploidy level and distribution range

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Abstract

Background and aims *Phragmites australis* is a wetland grass with high genetic variability, augmented by its cosmopolitan distribution, clonal growth form and large variation in chromosome numbers. Different ploidy levels and ecotypes differ in morphology and ecophysiological traits, and may possess different levels of phenotypic variation. The aim of this study was to quantify the natural variation in ecophysiological characteristics of *P. australis*, and to explore whether differences in ecophysiological traits can be related to ploidy levels or to the geographic origin of the clones.

Methodology Fifteen clones of *P. australis* from Europe and Asia/Australia, representing five ploidy levels (4*x*, 6*x*, 8*x*, 10*x* and 12*x*), were grown in a common garden design for 119 days. Plant growth and light-saturated rate of photosynthesis (P_{max}), stomatal conductance (g_s), water use efficiency (WUE) and concentrations of photosynthetic pigments and mineral ions in the leaves were measured.

Principal results The growth of the plants and most ecophysiological parameters differed significantly between clones. The mean maximum shoot height varied from 0.9 to 1.86 m, P_{max} from 9.7 to 27 μmol m^{-2} s^{-1}, g_s from 0.22 to 1.41 mol m^{-2} s^{-1} and WUE from 13 to 47 μmol mol^{-1}. The concentrations of chlorophylls did not vary significantly between clones, but the chlorophyll *a*/*b* ratio and the concentrations of total carotenoids did. The observed differences were not explained either by the ploidy level *per se* or by the geographic origin or phylogenetic relationships of the clones.

Conclusions Phylogeographic relationships in *P. australis* on a global scale do not mirror the environment where the adaptations have evolved, and high phenotypic variation among and within clones complicates comparative studies. Future studies aimed at explaining differences in plant behaviour between *P. australis* populations should be careful in the selection of target genotypes and/or populations, and should avoid generalizing their findings beyond the genotypes and/or populations studied.

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Introduction

Genetic diversity among individuals within a species is reflected in the presence of alleles in the gene pool, and hence different genotypes within populations (Ward *et al.* 2008). Such diversity can be identified by phenotypic traits, such as morphology or size. The wetland grass – common reed (*Phragmites australis*) – is a species with very high genetic variability (Lambertini *et al.* 2006, 2008) that is augmented by its cosmopolitan distribution, clonal growth form and the large variation in chromosome numbers (Brix 1999; Clevering and Lissner 1999). The species includes several ploidy levels with tetraploids (4*x*) being the predominant ploidy level in Europe, while octoploids (8*x*) are predominant in Australia and Asia (Clevering and Lissner 1999). Polyploids often occur together within the same population. A well-known mixed cytotype population is in the Danube Delta in Romania, with $2n = 4x$, 6*x*, 8*x* and 12*x* (Clevering and Lissner 1999; Hansen *et al.* 2007; Lambertini *et al.* 2008). Another mixed cytotype population is in Far East Russia, in Sakhalin Island, with $2n = 4x$, 6*x*, 8*x* and 10*x* (Clevering and Lissner 1999).

Polyploidy often leads to an overall increase in plant size (Stebbins 1971), and the Romanian octoploid *P. australis* has been reported to have taller and thicker shoots and larger leaves than the tetraploids (Hanganu *et al.* 1999; Pauca-Comanescu *et al.* 1999; Clevering *et al.* 2001). The length of stomatal guard cells also increases with increasing ploidy level, suggesting a correlation between cell and plant size; however, these differences do not affect photosynthetic gas exchange at different ploidy levels (Hansen *et al.* 2007). On the contrary, Saltonstall *et al.* (2007) found significant differences in guard cell size and stomatal density between American native and European introduced *P. australis* clones that could not be explained by differences in genome size. Also, significant differences in ecophysiological characteristics between populations with different ploidy levels, as well as within a ploidy level, have been reported (Kühl *et al.* 1999; Clevering *et al.* 2001; Lessmann *et al.* 2001; Hansen *et al.* 2007). Hence, there is plenty of evidence that large genetically determined differences in morphology and physiology exist among populations and clones of *P. australis*.

In *P. australis*, reproduction occurs both by vegetative spreading of shoots and rhizomes and long-distance dispersal of seeds (Haslam 1973), and several studies have documented large genetic variability within local *P. australis* populations (Lambertini *et al.* 2008; Fer and Hroudova 2009). However, at a global scale, *P. australis* can be grouped into a number of genetically related groups (Lambertini *et al.* 2006). The North American native population of *P. australis*, formally recognized as *P. australis* ssp. *americanus* (Saltonstall *et al.* 2004), constitutes one well-defined group. Another distinctive *P. australis* group extends throughout East Asia and Australia, and is predominantly comprised of octoploids (Lambertini *et al.* 2006). Tetraploids are also present in the Far East, but these are genetically more closely related to European *P. australis* than to the Asiatic/Australian group (Lambertini *et al.* 2006). The European *P. australis* ssp. *australis* (Clayton 1968) belongs genetically to a very diverse group with large cytological variation and an almost cosmopolitan distribution (Lambertini *et al.* 2006).

This study focuses on a comparison of ecophysiological traits between seven European clones and eight Asiatic/Australian clones with different ploidy levels. Unlike previous studies, we have considered all cytotypic variation present in Europe and Asia/Australia, and compared the two geographic ranges dominated by tetraploids and octoploids, respectively. The aim was to assess the natural variation in ecophysiological characteristics that occurs between them, and to explore whether consistent differences in ecophysiological traits can be related to the ploidy level and/or the geographic distribution range. Differences in ecophysiological traits might have evolved as a result of adaptation to the environmental conditions in the different geographic ranges, eventually in concert with polyploidization.

Materials and methods

Plant material

Fifteen clones of *P. australis* (Cav.) Trin ex Steud. from different geographic ranges (Europe and Asia/Australia) and with different ploidy levels ($2n = 4x$, 6*x*, 8*x*, 10*x*, 12*x*) were used in this study (Table 1). The clones were a subset of the clones used by Lambertini *et al.* (2006) to study the phylogeographic relationships of the *Phragmites* genus. The clones were selected to represent the geographic distribution of cytological variation of the species, and included clones from both mono- and mixed-cytotype populations. Two clones of each ploidy level were chosen to represent the European versus the Asiatic/Australian variation. However, dodecaploids (12*x*), which occur only in Europe (Clevering and Lissner 1999), were represented by only a single clone, and tetraploids (4*x*) only by a single clone in Asia/Australia. The Asiatic/Australian group comprised three octoploid (8*x*) clones (from Japan, Australia and Sakhalin Island in Russia) in an attempt to cover the distribution range of the Asiatic/Australian octoploid group. Decaploids (10*x*), which occur only in Asia/Australia (Clevering and Lissner 1999), were represented by clones from

Table 1 List of *P. australis* clones used in the study, their origin, ploidy level and phylogeographic relationships. Sample labels are the same as in Lambertini *et al.* (2006), but the prefix 'Pa', standing for *P. australis*, has been replaced by 'E' and 'A', indicating the geographic distribution of the clones in Europe (E) or Asia/Australia (A).

Sample label	Origin	Ploidy level	Phylogeographic relationships*
E646RO4x	Romania, Lake Razim	4*x*	*P. australis* core group
E620CZ4x	Czech Republic, Rozmberk	4*x*	*P. australis* core group
E625RO6x	Romania, Lake Oborny	6*x*	*P. australis* core group
E656RO6x	Romania, Lake Razim	6*x*	*P. australis* core group
E624RO8x	Romania, Lake Obretinu	8*x*	*P. australis* core group
E666CZ8x	Czech Republic	8*x*	*P. australis* core group
E660RO12x	Romania, Lake Razim	12*x*	*P. australis* core group
A205RU4x	Russia, Sakhalin, Novikovo	4*x*	*P. australis* core group
A139RU6x	Russia, Sakhalin, C. Maguntan	6*x*	Unknown
A213RU6x	Russia, Sakhalin, Voskhod	6*x*	*P. australis* Australia – E Asia
A120JP8x	Japan, Okoyama	8*x*	*P. australis* Australia – E Asia
A136AU8x	Australia, SA, Cortina Lake	8*x*	*P. australis* Australia – E Asia
A215RU8x	Russia, Sakhalin, Pokrovka, Nayba	8*x*	*P. australis* Australia – E Asia
A62RU10x	Sakhalin, Beregovoe	10*x*	*P. australis* Australia – E Asia
A133AU10x	Australia, NSW, Murrumbidgee River	10*x*	*P. australis* Australia – E Asia

*Lambertini *et al.* (2006).

Russia and New South Wales (Australia). The ploidy levels were determined by flow cytometry (Clevering and Lissner 1999). Phylogenetically, the clones belonged to the '*P. australis* core group', which is a large and mostly tetraploid ($2n = 4x$) group dominating in Europe and in North America as an invasive species, and the '*P. australis* Australia–E Asia group', which comprises octo- and decaploid clones from Australia and tropical and temperate East Asia (Lambertini *et al.* 2006).

In order to produce similar-sized, genetically identical plants for use in the experiment, shoots of the 15 clones were cut at their base and laid on a sand substrate in a 20- to 30-mm water layer, in a heated greenhouse for 1 month, until new shoots with roots were produced at the stem nodes of the layered shoots. The shoots were taken from a live collection of *P. australis* clones kept at the Department of Bioscience, Aarhus University, Denmark (56°13N; 10°07E), for at least 5 years prior to the study. Ten genetically identical shoots of each clone were then planted in five 3.5-L plastic pots in commercial compost, with two plants in each pot. The pots were watered with a fertilizing solution prepared from tap water and a commercial nutrient solution (100 mg L^{-1} Pioner NPK Makro 19-2-15+Mg and 0.1 ml L^{-1} Pioner Mikro+Fe, Brøste, Lyngby, Denmark). In order to maintain equal water levels in all pots, each pot was placed in a 6-L outer container filled with fertilizing solution to a height of ~100 mm. The smallest plant was removed from the pots after the first 14 days of establishment in the greenhouse. The whole experimental set-up comprised 15 clones, each represented by five replicates. The clones were grown for 119 days under identical environmental conditions in a greenhouse. The plants were watered every third or fourth day as needed, and their relative position in the greenhouse was changed weekly to minimize the effects of environmental gradients within the greenhouse.

Environmental conditions

Air temperature, relative humidity and photosynthetically active radiation in the greenhouse were monitored by a combined temperature and humidity sensor (Rotronic MP100TS-000; Bassersdorf, Switzerland) and an LI-190 Quantum Sensor (Li-Cor Biosciences, Lincoln, NE, USA), and all data were stored by an LI 1400 datalogger (Li-Cor Biosciences). The monthly average air temperature fluctuated from a maximum of 22 °C at noon to 14 °C at midnight in July and August, and from 20 to 11 °C in September and October. The relative air humidity fluctuated between 30 and 95 %, with strongest variations in July. Relative humidity values below 50 % were rare and occurred only in July. The average light intensity

during the daytime was highest in July, with a maximum of 934 μmol m^{-2} s^{-1} on 26 July. Thereafter, the light intensity decreased, reaching peak values of 635, 427 and 183 μmol m^{-2} s^{-1} in August, September and October, in concert with the decrease in day length.

Plant growth

Plant height (i.e. distance from the soil surface to the apical node of the tallest shoot in each pot) was measured at the beginning of the experiment and every second or third week. The shoot elongation rate (in millimetres per day) was calculated from the height difference between two successive samplings until shoot elongation stopped.

Gas exchange and chlorophyll fluorescence

After 42–49 days, when all plants were in an active growth phase, the light-saturated rate of photosynthesis (P_{max}), transpiration and stomatal conductance (g_s) were measured on the third or fourth youngest fully developed leaf of each plant, using an LI-6400XT Portable Photosynthesis System (Li-Cor Biosciences). Leaves were acclimated in the leaf chamber for 3–5 min until steady-state gas exchange properties were observed. The leaf chamber was air conditioned at 20 °C with a relative humidity of 35–50 %, and placed on a tripod to ensure stability during readings. The leaf chamber was supplied with atmospheric air drawn from a height of 5 m from outside the greenhouse. Light was supplied by an LI-6400-02B LED light source (Li-Cor Biosciences) set at 1800 μmol m^{-2} s^{-1} intensity. The leaf width was measured prior to infra-red gas-exchange analysis (IRGA) measurements to estimate the leaf area in the chamber. P_{max} was registered when the IRGA showed stable readings, usually after 2–5 min. The intrinsic water use efficiency (WUE) was calculated as P_{max} divided by g_s. The potential quantum yield of photosystem II (PSII), F_v/F_m, was measured in a dark-acclimated leaf from each replicate with a Portable Chlorophyll Fluorometer (PAM-2000, Walz Mess- und Regeltechnik, Germany). The saturation pulse method was used. The leaf was wrapped in aluminium foil for at least 15 min before a Walz Dark Leaf Clip (DLC-8) was used, together with the PAM, to take the readings of the F_v/F_m ratios.

Chlorophyll analyses

The leaves used in photosynthetic gas exchange measurements were frozen, and then lyophilized. Concentrations of chlorophylls (Chl *a*, Chl *b*) and total carotenoids (Total-car) were measured in a spectrophotometer after extraction of ~5 mg of leaf dry weight (dw) in 8 mL of 96 % ethanol according to Lichtenthaler (1987). Total-car is xanthophyll (x) plus carotenes (c). Pigment concentrations were expressed as milligrams per gram dry weight, and the ratios between the concentration of total chlorophylls and carotenoids, $[(a + b)/(x + c)]$, were calculated.

Water-extractable ions

At the end of the experiment, plants were harvested and the leaves frozen and lyophilized. The freeze-dried leaves were ground and ~0.1 g dw of ground plant material was extracted in 30-mL centrifuge tubes with 15 mL of Milli-Q water (Millipore) at 80 °C for 20 min. After cooling, 15 mL of Milli-Q water were added and the samples centrifuged for 5 min at 1700 *g*. The concentrations of chloride (Cl) in the extractions were determined by titration with $AgNO_3$ (ABU52 Biburette Titrator, TitraMaster 85, Radiometer Analytical SAS, France). Concentrations of sodium (Na), potassium (K), calcium (Ca) and magnesium (Mg) in the extracts were analysed by ICP-OES (Optima 2000 DV, Perkin Elmer Instruments Inc., CT, USA).

Statistics

Differences in the measured parameters due to geographic range (GR), ploidy level (PL) and clonal variation were analysed by a nested ANOVA using the GLM procedure of the software Statgraphics Centurion XV (Manugistics Inc., MD, USA). Geographic range and ploidy level were treated as independent factors, whereas clonal variation was nested within GR × PL. Outliers were identified by the unusual residual procedure. Values with residuals >3.5 were eliminated. Differences among clones in the measured parameters were identified by one-way ANOVAs. Data were tested for variance homogeneity using Levene's test, and if necessary log transformed. The post hoc Tukey's HSD (honestly significant difference) test was used to identify significant differences among the clones at the 95 % confidence level. A rotated principal component analysis (PCA) was conducted on the measured ecophysiological characteristics of the 15 clones, in order to reduce the number of variables into a smaller number of principal components that account for most of the variance in the data.

Results

Differences due to geographic range and ploidy level

The two-way nested ANOVA did not detect statistically significant differences in any of the measured ecophysiological characteristics between geographic origin or ploidy level (Table 2). The proportion of the variability that could be related to the geographic origin and the ploidy level was generally much lower than the

Table 2 Summary of a two-way ANOVA showing the effects of geographic range (GR) and ploidy level (PL) on the measured ecophysiological parameters in *P. australis* clones. The factor 'clone' was nested in 'GR × PL' in the ANOVA. Variance within each factor was tested against the total variation of the clones, each represented by its five replicates. SS % = sum of squares as a percentage; df = degrees of freedom. Numbers in boldface indicate *P* values <0.05.

Parameter	GR (df = 1)		PL (df = 4)		Clone (GR × PL) (df = 7)		Residual (df = 51–59)
	SS %	*P*	SS %	*P*	SS %	*P*	SS %
Shoot height	2.9	0.594	7.2	0.934	64.5	**0.000**	27.0
Photosynthesis (P_{max})	32.2	0.066	24.8	0.505	47.3	**0.000**	24.4
Stomatal conductance (g_s)	8.2	0.351	20.6	0.659	57.5	**0.000**	18.6
WUE	0.1	0.934	21.1	0.602	50.8	**0.000**	16.7
F_v/F_m ratio	0.3	0.809	18.6	0.398	27.6	**0.001**	53.5
Chl $a + b$	0.4	0.664	14.8	0.186	12.4	0.209	71.4
Chl a/b ratio	0.8	0.732	3.1	0.972	46.4	**0.000**	49.3
Total-Car	3.1	0.460	12.9	0.649	35.2	**0.000**	48.1
$[(a + b)/(x + c)]$	0.0	0.981	17.3	0.789	71.8	**0.000**	15.2
Cl	2.9	0.590	14.5	0.805	63.9	**0.000**	21.2
Ca	8.9	0.142	31.1	0.150	22.9	**0.001**	45.5
K	0.8	0.634	46.3	0.061	21.5	**0.000**	29.0
Mg	17.5	0.110	28.0	0.344	36.5	**0.000**	31.7
Na	6.7	0.370	9.6	0.850	50.8	**0.000**	27.4

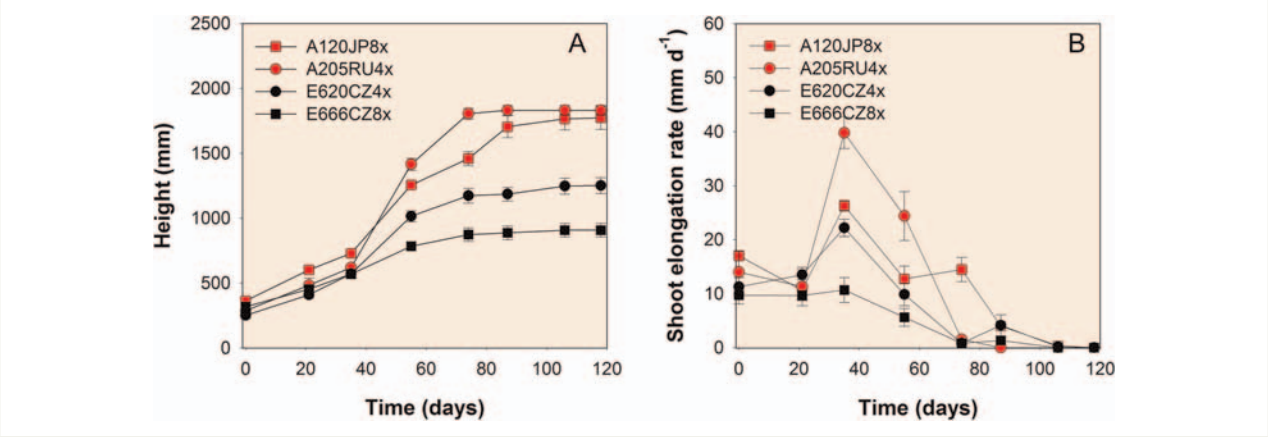

Fig. 1 Average shoot height (A) and shoot elongation rate (B) of four significantly different *P. australis* clones over time. The clones were propagated from stem nodes of layered shoots and grown in a greenhouse for 119 days. Clones shown in the figure represent *P. australis* from Europe and Asia/Australia and two ploidy levels (4*x* and 8*x*). Data for only four clones are shown to simplify the graphs.

variability between clones within the geographic range and the ploidy level. Hence, the variation in the data set appeared to be explained largely by differences among clones. P_{max} was the parameter closest to being affected by geographic range ($P = 0.066$) with slightly—but not statistically significant—higher rates in the Asiatic/Australian clones than the European clones. For ploidy levels, K concentration was the parameter closest to being affected ($P = 0.061$), but again this was not statistically significant.

Table 3 Average final shoot height, light-saturated rate of photosynthesis (P_{max}), stomatal conductance (g_s), intrinsic water use efficiency (WUE) and potential quantum yield of PSII (F_v/F_m) of *P. australis* clones with different ploidy levels, and from two different geographic ranges as indicated by the first letter in the clone name ('E', Europe; 'A', Asia/Australia).

Clone	Shoot height (cm)	P_{max} (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	WUE (μmol mol^{-1})	F_v/F_m
E646RO4x	165 ± 13^{def}	15.8 ± 2.0^{bc}	0.70 ± 0.37^{bcde}	27.4 ± 2.7^{cdef}	0.79^{abc}
E620CZ4x	125 ± 13^{abc}	21.8 ± 2.0^{cd}	0.51 ± 0.12^{bc}	44.0 ± 2.4^{h}	0.79^{bc}
E625RO6x	158 ± 10^{cdef}	20.7 ± 2.6^{cd}	1.12 ± 0.11^{efg}	18.4 ± 2.4^{bcd}	0.80^{bc}
E656RO6x	140 ± 26^{bcd}	9.7 ± 1.3^{a}	0.38 ± 0.07^{ab}	25.4 ± 3.1^{cdefg}	0.74^{a}
E624RO8x	181 ± 26^{ef}	19.2 ± 3.8^{c}	1.41 ± 0.38^{h}	13.6 ± 2.4^{a}	0.78^{abc}
E666CZ8x	90 ± 11^{a}	10.5 ± 0.6^{ab}	0.22 ± 0.03^{a}	47.2 ± 3.8^{gh}	0.82^{bc}
E660RO12x	147 ± 23^{bcde}	16.9 ± 1.1^{c}	0.49 ± 0.09^{abcd}	34.8 ± 2.7^{fgh}	0.80^{c}
A205RU4x	180 ± 8^{ef}	27.0 ± 2.2^{d}	1.22 ± 0.15^{gh}	22.1 ± 2.7^{bcde}	0.80^{bc}
A139RU6x	112 ± 12^{ab}	26.6 ± 3.9^{d}	1.02 ± 0.29^{efg}	26.7 ± 2.4^{cdefg}	0.79^{bc}
A213RU6x	113 ± 7^{ab}	19.8 ± 1.9^{cd}	0.95 ± 0.14^{defg}	20.8 ± 2.7^{bcde}	0.75^{ab}
A120JP8x	177 ± 19^{ef}	18.1 ± 1.8^{c}	1.24 ± 0.33^{fgh}	15.4 ± 2.4^{b}	0.80^{c}
A136AU8x	134 ± 10^{bcd}	21.9 ± 2.6^{cd}	0.82 ± 0.13^{cdef}	27.1 ± 2.4^{defg}	0.79^{bc}
A215RU8x	113 ± 10^{ab}	16.6 ± 2.6^{c}	0.54 ± 0.13^{bcd}	30.9 ± 2.4^{efgh}	0.78^{abc}
A62RU10x	122 ± 13^{ab}	18.2 ± 4.2^{c}	0.43 ± 0.16^{ab}	43.9 ± 2.4^{h}	0.80^{bc}
A133AU10x	186 ± 14^{f}	15.5 ± 4.4^{bc}	0.38 ± 0.10^{ab}	40.3 ± 3.1^{fgh}	0.80^{bc}

Means ± SD are shown; different letters within columns indicate significant differences ($P < 0.05$) between clones.

Differences among clones

Growth Differences in size and growth rate between the clones were present during the first month of the experiment. The average height of the plants at the time of planting ranged from 202 mm (A139RU6x) to 388 mm (E624RO8x), even though all clones were propagated at the same time. Some clones reached their maximum height earlier than others (Fig. 1A). At the end of the experiment, the maximum height ranged from 900 mm (E666CZ8x) to 1860 mm (A205RU4x) (Table 4). The tallest clones had different ploidy levels and geographic range (A133AU10X, E624RO8x, A205RU4x, A120JP8x, E656RO6x and E625RO6x) and the shortest clone was a European octoploid (E666CZ8x) (Table 3). The shoot elongation rate also varied significantly between clones and over time (Fig. 1B).

Physiological parameters Significant differences in P_{max}, g_s and WUE, as well as F_v/F_m ratio were found among the clones (Table 3). The P_{max} varied from 9.7 μmol CO_2 m^{-2} s^{-1} in a hexaploid European clone (E656RO6x) to 27 μmol CO_2 m^{-2} s^{-1} in a tetraploid Asiatic clone (A205RU4x). Consistent differences in P_{max} between European and Asiatic/Australian clones, as indicated by the distribution of variance (Table 2), could not be directly observed in the data. Two Asiatic clones with high P_{max} values appeared to explain the non-significant difference between the two geographic ranges (Table 3). The g_s ranged from 0.22 to 1.41 mol m^{-2} s^{-1}, and WUE ranged between 13.6 and 47.2 μmol CO_2 mol^{-1} H_2O (Table 3). The highest and lowest g_s, and correspondingly lowest and highest WUE, were found in European octoploids (E666CZ8x and E624RO8x). Although significant differences in the F_v/F_m ratio were observed among clones, the ratios were relatively constant, i.e. restricted to a range of 0.74–0.82.

Chlorophyll and carotenoids The concentrations of Chl *a*, Chl *b* and Chl_{a+b} did not differ significantly between the clones. Chl *a* and Chl *b* concentrations ranged from 4.70 to 6.67 and 1.56 to 2.39 mg g^{-1} dw, respectively (Table 4). Significant differences were observed in the Chl *a*/*b* ratio, as well as in the concentrations of Total-car and the ratio between Chl_{a+b} and Total-car, $[(a+b)/(x+c)]$ ratio (Table 4). Two Romanian hexaploids (E625RO6x and E656RO6x) had the highest and the lowest values for the Chl *a*/*b* ratio, respectively, whereas the minimum and the maximum Total-car concentrations and, inversely, $[(a+b)/(x+c)]$ ratios, were among Asiatic clones (A120JP8x and A139RU8x).

Water-extractable ions The concentrations of Cl, Na, K, Ca and Mg in the leaves of the plants differed significantly

Table 4 Average chlorophyll and carotenoid (Total-Car) concentrations, ratio of Chl *a* to Chl *b*, and the Chl_{a+b} to total carotenoid ratio [(*a* + *b*)/(*x* + *c*)] of *P. australis* clones with different ploidy levels, and from two different geographic ranges as indicated by the first letter in the clone name ('E', Europe; 'A', Asia/Australia).

Clone	Chl *a* (mg g^{-1} dw)	Chl *b* (mg g^{-1} dw)	Chl_{a+b} (mg g^{-1} dw)	Chl *a*/*b* ratio	Total-Car (mg g^{-1} dw)	[(*a* + *b*)/(*x* + *c*)]
E646RO4x	5.89 ± 0.93	2.09 ± 0.27	7.99 ± 1.20	2.81 ± 0.12^{bcd}	0.96 ± 0.17^{bcd}	8.28 ± 0.82^{abc}
E620CZ4x	6.66 ± 1.15	2.39 ± 0.36	9.06 ± 1.51	2.77 ± 0.12^{abcd}	0.84 ± 0.17^{abcd}	10.71 ± 0.80^{c}
E625RO6x	5.52 ± 0.79	2.33 ± 0.18	7.85 ± 0.97	2.35 ± 0.18^{a}	0.62 ± 0.25^{abc}	10.85 ± 1.57^{cd}
E656RO6x	5.50 ± 0.87	1.77 ± 0.35	7.27 ± 1.22	3.12 ± 0.20^{d}	1.02 ± 0.08^{cd}	6.87 ± 0.69^{a}
E624RO8x	4.79 ± 0.31	1.56 ± 0.13	6.36 ± 0.44	3.06 ± 0.15^{d}	0.79 ± 0.08^{abcd}	8.01 ± 1.09^{abc}
E666CZ8x	5.61 ± 0.83	2.15 ± 0.26	7.76 ± 1.08	2.60 ± 0.20^{abc}	0.55 ± 0.12^{ab}	13.98 ± 1.78^{d}
E660RO12x	4.99 ± 0.63	1.75 ± 0.16	6.74 ± 0.77	2.83 ± 0.20^{bcd}	0.65 ± 0.17^{abc}	9.80 ± 1.50^{bc}
A205RU4x	5.59 ± 1.24	1.87 ± 0.41	7.46 ± 1.63	2.99 ± 0.28^{cd}	0.82 ± 0.20^{abcd}	9.07 ± 1.38^{abc}
A139RU6x	6.67 ± 0.59	2.23 ± 0.23	8.91 ± 0.80	2.99 ± 0.13^{cd}	1.08 ± 0.12^{d}	8.12 ± 0.38^{abc}
A213RU6x	6.03 ± 0.94	2.14 ± 0.25	8.17 ± 1.16	2.79 ± 0.23^{abcd}	0.97 ± 0.22^{cd}	8.42 ± 1.11^{abc}
A120JP8x	4.70 ± 0.99	1.85 ± 0.39	6.56 ± 1.37	2.54 ± 0.18^{ab}	0.44 ± 0.19^{a}	19.23 ± 2.23^{e}
A136AU8x	5.55 ± 0.89	1.90 ± 0.23	7.45 ± 1.08	2.90 ± 0.19^{bcd}	0.87 ± 0.23^{bcd}	8.68 ± 1.39^{abc}
A215RU8x	5.79 ± 1.03	2.13 ± 0.30	7.92 ± 1.32	2.71 ± 0.16^{abcd}	1.03 ± 0.17^{cd}	7.73 ± 1.50^{ab}
A62RU10x	5.80 ± 1.01	2.06 ± 0.34	7.87 ± 1.35	2.81 ± 0.07^{abcd}	0.91 ± 0.16^{bcd}	8.30 ± 0.16^{abc}
A133AU10x	6.28 ± 0.79	2.31 ± 0.19	8.06 ± 0.93	2.71 ± 0.24^{abcd}	0.80 ± 0.21^{abcd}	9.78 ± 1.19^{bc}

Means ± SD are shown; different letters within columns indicate significant differences ($P < 0.05$) between clones.

Table 5 Average water-extractable ion concentrations in leaves of *P. australis* clones with different ploidy levels, and from two different geographic ranges as indicated by the first letter in the clone name ('E', Europe; 'A', Asia/Australia).

Clone	Cl (μmol g^{-1} dw)	Na (μmol g^{-1} dw)	K (μmol g^{-1} dw)	Ca (μmol g^{-1} dw)	Mg (μmol g^{-1} dw)
E646RO4x	496 ± 70^{fgh}	71 ± 9^{c}	427 ± 62^{ab}	177 ± 26^{bc}	51 ± 12^{abcd}
E620CZ4x	222 ± 40^{a}	29 ± 6^{a}	319 ± 42^{a}	121 ± 37^{ab}	43 ± 7^{abc}
E625RO6x	401 ± 44^{cdefgh}	35 ± 12^{a}	422 ± 58^{ab}	135 ± 29^{ab}	54 ± 9^{bcd}
E656RO6x	410 ± 45^{cdefgh}	37 ± 3^{ab}	549 ± 100^{bc}	147 ± 23^{abc}	74 ± 16de
E624RO8x	372 ± 48^{bcd}	26 ± 9^{a}	500 ± 37^{bc}	163 ± 30^{abc}	84 ± 15^{e}
E666CZ8x	548 ± 32^{h}	54 ± 12^{bc}	553 ± 91^{bc}	154 ± 37^{abc}	59 ± 9^{bcde}
E660RO12x	523 ± 45^{gh}	37 ± 13^{ab}	776 ± 61^{d}	118 ± 45^{ab}	65 ± 6^{cde}
A205RU4x	480 ± 43^{efgh}	29 ± 2^{a}	620 ± 68^{cd}	103 ± 15^{a}	41 ± 5^{abc}
A139RU6x	302 ± 48^{ab}	31 ± 5^{a}	475 ± 113^{abc}	115 ± 13^{ab}	29 ± 4^{a}
A213RU6x	486 ± 43^{efgh}	29 ± 6^{a}	620 ± 57^{cd}	103 ± 13^{a}	41 ± 12^{abc}
A120JP8x	456 ± 50^{defgh}	28 ± 5^{a}	400 ± 110^{ab}	169 ± 44^{abc}	82 ± 17^{e}
A136AU8x	381 ± 31^{bcde}	31 ± 8^{a}	547 ± 36^{bc}	115 ± 35^{ab}	35 ± 5^{ab}
A215RU8x	349 ± 60^{bc}	31 ± 3^{a}	316 ± 65^{a}	105 ± 20^{a}	64 ± 9^{cde}
A62RU10x	477 ± 68^{defgh}	34 ± 5^{a}	446 ± 80^{ab}	160 ± 38^{abc}	57 ± 9^{abcde}
A133AU10x	392 ± 9^{bcdef}	30 ± 4^{a}	412 ± 46^{ab}	204 ± 19^{c}	59 ± 12^{bcde}

Means ± SD are shown; different letters within columns indicate significant differences ($P < 0.05$) between clones.

between the clones (Table 5). Leaf tissue concentrations of Cl and Na ranged from 222 to 548 and 26 to 71 μmol g^{-1} dw, respectively. Minimum and maximum Cl concentrations were found in the tissues of two Czech clones (E620CZ4x and E666CZ8x), and minimum and maximum Na concentrations were in two Romanian clones from the Danube Delta (E624RO8x and E646RO4x). Differences in the concentrations of Ca and Mg due to geographic range (Table 3) were not obvious when all clones were analysed individually, and were better explained by a function of individual clones rather than any general geographic trend. The Ca concentration ranged from 103 to 204 μmol g^{-1} dw among Asiatic clones with different ploidy levels. Magnesium ranged from 29 to 84 μmol g^{-1} dw in an Asiatic hexaploid and a European octoploid. The K concentration ranged from 316 to 620 μmol g^{-1} dw among Asiatic clones with different ploidy levels.

Principal component analysis The PCA extracted four factors with eigenvalues >1. Together they accounted for 81.9 % of the variation in the original data (Table 6). Factor 1 (PC1) had high negative loadings for Total-car and the Chl *a*/*b* ratio, and positive loading for the ratio between Chl_{a+b} and Total-car, [$(a + b)/(x + c)$], and could be interpreted as a pigment-related factor. Factor 2 had high loadings for shoot height, photosynthesis, stomatal conductance and WUE, and could be interpreted as a growth-related factor. Factor 3 had high loadings for Cl, K and Na, and factor 4 had high loadings for Ca and Mg, and could be interpreted as a monovalent-ion-related and a divalent-ion-related factor, respectively. The plots of factor scores against each other (Fig. 2) did not group the *P. australis* clones according to the ploidy level or geographic range. However, with the exception of the Asiatic clone from Japan (A120JP8x), the clones of the Asiatic/Australian group clustered close to each other, particularly for the pigment-related factor (PC1), indicating similar ecophysiological characteristics, whereas the European clones spread widely in the PCA, suggesting a high phenotypic variation in ecophysiological traits within European *P. australis*.

Table 6 Results of PCA of the 12 measured parameters that differed significantly between the 15 *P. australis* clones in the post hoc Tukey test. Factor 1 is a 'pigment-related factor', factor 2 a 'growth-related factor', factor 3 a 'monovalent ion-related factor' and factor 4 a 'divalent ion-related factor'.

	Eigenvalue	Proportion of variance (%)	Cumulative proportion of variance (%)	
PC1	3.74	31.2	31.2	
PC2	2.72	22.7	53.8	
PC3	1.90	15.9	69.7	
PC4	1.46	12.1	81.9	
Factor loading matrix after Varimax rotation				
	PC1	PC2	PC3	PC4
Shoot height	0.053	**0.605**	0.254	0.575
Photosynthesis (P_{max})	−0.144	**0.629**	−0.347	−0.268
Stomatal conductance (g_s)	0.087	**0.969**	−0.131	−0.051
WUE	0.019	**−0.899**	−0.034	0.022
Total-Car	**−0.868**	−0.123	−0.278	−0.226
Chl *a*/*b* ratio	**−0.900**	0.145	0.230	−0.037
$(a + b)/(x + c)$	**0.780**	0.087	0.025	0.351
Cl	0.317	0.015	**0.891**	0.141
Ca	0.482	−0.443	0.022	**0.613**
K	−0.348	−0.132	**0.848**	−0.001
Mg	0.171	−0.121	0.035	**0.833**
Na	**0.633**	−0.147	0.491	−0.422

Variables with high loadings are shown in bold.

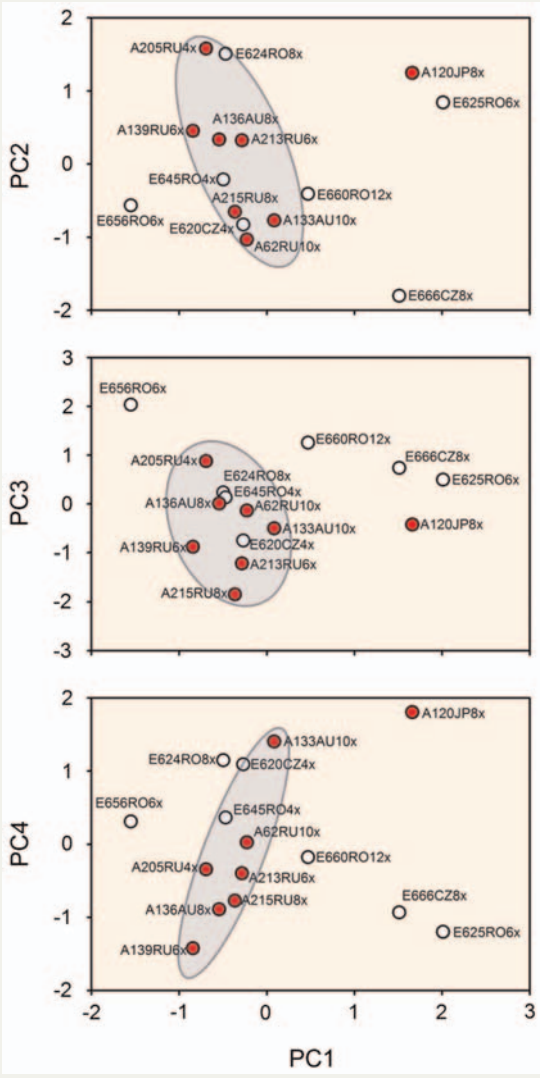

Fig. 2 Factor score plots from a rotated PCA on the measured ecophysiological characteristics of 15 *P. australis* clones. Factor 1 is a 'pigment-related factor', factor 2 a 'growth-related factor', factor 3 a 'monovalent ion-related factor' and factor 4 a 'divalent ion-related factor'. Red dots represent Asiatic/Australian clones, and white dots European clones. Shaded circles delineate the Asiatic/Australian clones, except for clone A120JP8x. The similarities among the Asiatic/Australian clones do not fully match their phylogeographic relationships, as clone A205RU4x is more closely related to the European group than to the Asiatic/Australian group, whereas clone A120JP8x belongs to the Asiatic/Australian group (Lambertini *et al.* 2006).

Discussion

The aim of this study was to evaluate the extent to which differences in the ploidy level and geographic range within the cosmopolitan grass *P. australis* are reflected in phenotypic traits, such as plant size and ecophysiological characteristics, and if the predominance of octoploids in Asia and Australia could be explained by a superior phenotype that may have evolved within the highly genetically and cytotypically diverse *P. australis* species. Hence, we grew clones of different cytotypes collected in Europe (where $2n = 4x$ is the dominant ploidy level) and in Asia and Australia (where octoploids predominate) under identical environmental conditions to avoid environmental effects on phenotype development. Furthermore, the clones had been grown in a common outdoor environment under similar conditions in terms of soil, water, nutrition and climate for at least 5 years prior to the experiment. It is therefore justified to assume that any difference in growth, morphology and physiological characteristics between the clones is genetically determined.

Previous studies have reported significant variations in stand height, plant morphology and salinity tolerance related to the ploidy level (Hanganu *et al.* 1999; Pauca-Comanescu *et al.* 1999; Hansen *et al.* 2007). In the Danube delta, Romania, tetra- (4*x*), hexa- (6*x*) and octoploids (8*x*) co-exist, and tetra- and octoploids can be identified by their phenotype as shoots of octoploids are longer and thicker with more nodes than those of tetraploids, and panicles and leaves of octoploids are also larger than those of tetraploids (Pauca-Comanescu *et al.* 1999). Also, Hansen *et al.* (2007) found that different ploidy levels had different morphologies, with Romanian octoploids generally being taller and thicker than other ploidy levels. In the present study, another Romanian octoploid (E624RO8x) was also among the tallest clones, although not significantly taller than a Russian tetraploid, a Japanese octoploid or an Australian decaploid. Hence, it does not seem to be a general characteristic that octoploids are larger than other ploidy levels. Also, our results did not show any statistically significant differences between ploidy levels. For example, the Czech octoploid (E666CZ8x) was shorter than the corresponding Czech tetraploid (E620CZ4x), and in plants from the Danube delta, where different ploidy levels co-exist, there were no significant differences between the different ploidy levels (E646RO4x, E624RO8x, E625RO6x, E656RO6x and E660RO12x). Although European clones appeared very different in size and ecophysiological traits, the Asiatic and Australian clones were very similar. However, similarities were independent of ploidy level and phylogenetic relationships. Not all Asiatic/Australian clones analysed in the present study belonged to the predominantly octoploid group identified by Lambertini *et al.* (2006). The clone A205RU4x was genetically closer to the European clones than to the Asian/Australian octoploids, but it did not cluster

far from the Asiatic/Australian clones in the PCA. Although other European clones clustered together with the Asiatic/Australian clones, the Japanese octoploid clone clustered far away from its close relatives in Sakhalin Island and Australia, revealing large variation in ecophysiological traits also within the predominantly octoploid group that evolved in Asia and Australia.

Differences among clones could not be explained by the ploidy level, geographic range or phylogenetic relationships, but appeared to be genotype dependent. However, the variation was remarkably higher within the European group of clones than within the Asiatic/Australian group, considering that European clones were from a restricted geographic area (Romania and the Czech Republic), whereas the Asiatic/Australian clones covered a much larger geographic range represented by Sakhalin Island, Japan and Australia.

It has been suggested that growth and expansion rates are a combined effect of genotype and the native environment (Parker *et al.* 2003; Ward *et al.* 2008). This interaction underlines the possible features of successful invaders, which correlate with the length of the growing season – one of the opportunistic traits of invasive species (Zedler and Kercher 2004). The tetraploids analysed in the present study seemed, in general, to invest predominantly in the first stage of the growing season, with intensive growth in spring to reach the maximum height after 2–3 months. In contrast, growth rates of octoploids were moderate in spring, but growth extended over a longer period of time. This difference in growth pattern suggests that the clones are adapted to growing seasons of different length, and also that tetraploids possess more opportunistic traits than octoploids. Other studies also found differences in the length of the growing season among clones that could be related to the geographic origin of the clones (Clevering *et al.* 2001).

The 15 *P. australis* clones analysed in this study had high phenotypic variation in P_{max}, g_s and WUE. The variations observed were genotype dependent, but they could not be clearly attributed to the ploidy level or to the geographic range of origin. This was due to the high variability between representatives of the same ploidy level and variability between clones from the same geographic range, as well as variation between replicas of the same clone. Although all clones had origins at similar latitudes (lowest 35°, highest 50°) and had been acclimated to the same environment for at least 5 years prior to the experiment, significant differences in P_{max}, g_s and WUE were observed between clones. Hence, the clones did not acclimate to the growth environment to the same extent. Physiological processes have earlier been reported to acclimate to new growth environments (Lessmann *et al.* 2001). However, the native habitat of the clones and differential expression of photosynthesis-related genes may also be some of the reasons for the observed differences in gas exchange characteristics of the clones we studied. Environmentally induced differences in physiological parameters have been found to be generally larger than the genetically determined differences between populations of *P. australis* (Lessmann *et al.* 2001). Since in our experiment the premise of similar environmental conditions is ensured, the differences observed between clones can be assumed to be genetic.

The fact that the concentrations of the light-absorbing chlorophylls did not differ among clones is not surprising since the content of chlorophylls is expected to be rather stable within species under common environmental conditions. However, the observed difference in the Chl *a*/*b* ratio between the clones indicates variations of the functional pigment complex, as well as possible physiological differences in the photosynthetic apparatus. The concentration of total carotenoids as well as the chlorophyll to carotenoid ratio, $[(a + b)/(x + c)]$, also differed among the clones. These differences may be related to differences in environmental conditions and the length of the growing season in the native habitat of the clones, since these parameters are often associated with senescence (Kurahotta *et al.* 1987; Chen and Cao 2008).

The concentrations of Cl, Na, K, Ca and Mg in the leaves of the plants also differed significantly among the clones, and the differences could not be related either to the ploidy level or to the geographic origin of the clones. The concentrations of these elements in the leaves may be related to the osmotic balance of the cells, and hence to mechanisms and possible strategies adopted by the clones in stress conditions. A differential ability to regulate ions like Cl^- and Na^+ in the plant tissue may be related to differences in salt tolerance (Lissner and Schierup 1997; Lissner *et al.* 1999; Munns and Tester 2008; Pagter *et al.* 2009) and drought resistance (Pagter *et al.* 2005). The significant differences observed here support the hypothesis that stress tolerance in *P. australis* is genotype dependent.

Conclusions and forward look

The present study reveals significant genetically determined differences in a range of growth and ecophysiological traits between different *P. australis* genotypes, and provides evidence that the differences are not related either to the ploidy level *per se* or to the phylogeographic relationships of the genotypes. This finding is important in the context of those studies based on just a single genotype or population of a species, as well as studies

comparing different species based on only one population of each, as results may have been different if another set of populations was chosen. In addition to the genetically determined differences between the clones, the individual genotypes also showed high phenotypic variation, which further complicates studies with this species. The genotype-related variability in growth and ecophysiological traits may be a consequence of different strategies in relation to particular types of stress that have arisen as a result of selection, and the creation of local ecotypes with associated genetically determined ecophysiological characteristics. Our results broaden insights into the correlation between euploidy and ecophysiological traits within *P. australis*, as we found no general relationship. Hence, future studies aimed at explaining differences in plant behaviour need to be careful in the selection of target genotypes and/or populations, and should avoid generalizing their findings beyond the genotypes and/or populations studied.

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Contributions by the authors

L.A. carried out the controlled growth experiment and drafted the manuscript. C.L. and H.B. participated in the design of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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Conflict of interest statement

None declared.

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