Morphological and RAPD Variation of *Phragmites australis* along Salinity Gradient in the Wetlands of the Downstream of Yellow River, China†

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ABSTRACT : *Phragmites australis* is the dominant and constructive species among plant communities in the wetlands of the downstream of Yellow River, China. Its morphological characters were high variable in different habitats. Studies on Morphological and RAPD variation of 15 *P. australis* populations from this region showed that soil salinity was the dominant ecological factor that affected the morphological characters of *P. australis*. The basal diameter, height, leaf length, leaf width, internode length, internode accounts, panicle length were negatively related to salinity. 194 loci were amplified by RAPD, of which 9 loci was highly negative-related to salinity, and showed a tendency to prefer the habitats with fresh water. 4 loci were positively related to the salinity, and showed a tendency to prefer the salinized habitats. Most loci were neutral to salinity. The morphological and genetic characters of BZH were special, and the speciality should not be determined by salinity. The morphological characters were affected by genetic information and environment. The morphological characters should change gradually and continuously along environmental gradient under plasticity, but should changed continuously or not in genetic control. The relevancies among quantitive characters, ecological factors and genetic variation in natural populations still will still be a focus and difficulty of ecological genetics of *P. australis* in the future.

Key words : Genetic variation, Morphological variation, *Phragmites australis*, RAPD

INTRODUCTION

Common reed (*Phragmites australis* (Cav.) Trin. ex Steudel) is a worldwide spread wetland species, and the dominant *P. australis* communities play an important role in maintaining and adjusting wetlands ecological function (Clevering *et al*. 1999). *P. australis* shows high ecological adaptation and morphological variability in the different habitats. The morphological characters of *P. australis* were affected not only by water level, salinity (Zheng *et al*. 1999) and nutrition (Clevering 1998) etc, but also chromosome ploidy (Pauca-Comanescu *et al*. 1999), clonal speciality (Kùhl *et al*. 1999) and ecotypes variation (Clevering *et al*. 1999). The affection of salinity to seed germination was reported (Waisel and Rechav 1971). The affection of water depth to *P. australis* growth also has been investigated (Vretare *et al*. 2001). The relationship between expandability and genetic variation of *P. australis* in North America was reported (Pellegrin and Hauber 1999). But what leads to high adapatation of *P. australis* in the field? Is it morphology plasticity, or genetic variation? In this paper, we showed that it was a proper approach to these disputes to make clear the relation among morphological, genetic variation of *P. australis* and ecological factor such as salinity.

In the downstream of Yellow River within Shandong Province, China, there are a lot of kinds of wetlands formed by flood or drainage. Some wetlands are permanent flood, for example salt marsh, lake, riverbed, pond, etc (Zhang *et al*. 1993; Li 1994). Some wetlands are temporary flood, such as salinized meadow or grassland. *P. australis* is one of dominant and constructive species in this area. Soil salinity decreases gradually from estuary to inland due to the weakening of tide. It is helpful to perform researches on the relationship among salinity and morphological, genetic variation of *P. australis*. This research will aim at the soil salinity, an important ecological factor that affects the growth of *P. australis*, integrated with morphological investigation and RAPD analysis. For the aim we will try to solve two problems. First is to make clear the tendency of morphological and genetic along salinity grads. Second is to discuss the contribution of morphological and genetic to adaptation of *P. australis* to salinity.

All of the 7 morphological indexes as quantitive characters can

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reflect the profile that morphological characters varies gradually along salinity grads, and morphological differentiation level also can indirectly reflect the genetic differentiation among populations. RAPD as neutral marker is limited to explore the relationship between genetic variation and environment. It is difficult to mark the genes related to ecological adaptation because there is little knowledge about genome information of *P. australis*. And the finite number of enzyme systems and loci can be provided by allozymes. Therefore RAPD is an alternative approach to analyze quite a lot of loci. Koppitz (1999) had already used RAPD to study the clonal diversity and genetics structure of *P. australis*. The research tried to distinguish those loci related to the salinity from a great deal of loci by RAPD. Although this work was very difficult and apparently not too smart, it still gave us some fresh and meaningful information.

MATERIALS AND METHODS

Sampling sites

Fifteen natural *P. australis* stands from estuary to inland have selected from April 2000 to October 2000 according to salinity gradient and with a little disturbance. The geographic position of every site was located by GPS. Relative positions of all sites were shown in Fig. 1. The longitude and latitude, habitat character, growth situation of *P. australis* of every site were listed in Table 1. The

Fig. 1. The map with a scale of 1: 4 mi (below) showed the distribution of 15 sampling sites in the wetland of the downstream of Yellow River within Shandong province, China. The upper illustration is the enlargement of Yellow River Delta.

sites were arranged according to the order of actual investigation time, roughly from estuary to inland. Among 15 sites, HS01~LZH

Table 1. Location and the habitat characters of sampling sites

Code	Populations	Position of site		Growth situation	Remark
		N (°)	E (°)		
01	HS01	37.760	119.156	Good	
02	HS02	37.758	119.161	Less suppressed	
03	HS03	37.754	119.171	Seriously suppressed	
04	HS04	37.748	119.127	Few crawlling plants	
05	HS05	27.749	119.127	Slender	Yellow River Delta
06	DWL	37.761	118.985	Less suppressed	
07	KD	37.761	118.851	Thick and patch	
08	GD	37.771	118.664	High and dense	
09	LZ	37.791	118.544	Thin and dense	
10	LJ	37.521	118.269	Thin and dense	
11	BZH	37.444	117.943	High, thick and sparse	
12	DLH	37.230	117.929	Good and dense	Inland wetland along Yellow River
13	ZHG	36.486	116.567	High and dense	
14	DPH	36.035	116.217	High and dense	
15	NYH	35.311	116.627	High and dense	Freshwater lake

were located in the Yellow River Delta, LJ~DPH were in inland wetlands alongside the Yellow River, NYH was far away from the Yellow River, and was located in the Nanyang Lake, belonging to Huai He River basin and connecting to the Yellow River via the Grant Canal.

Salinity measurement

Three samples of soil with each section of 15cm were collected randomly from each sampling site in May 2000, respectively, when the soil is crucial to reed growth and development. Three samples of mud under the water were collected in flood areas. The total salinity of each soil sample was measured through the method of drying, weighting, filtrating, drying and re-weighting. The total weight of dry soil was weighed up as W_1. Then it was filtrated and then dissoluble salt and water were discarded. The soil was dried again and re-weighted up as W_2. Salinity, or gross dissoluble salt, was $(W_1-W_2)/W_1\times100\%$. The average salinity of 3 soil samples in every site was calculated (Institute of Soil Science, Chinese Academy of Sciences 1978; Guo *et al*. 2003).

Investigation of morphological characters

When the vegetative growth of *P. australis* had already stopped but had not died away in Oct. 2000, the basal diameter (mm), height (m), leaf length (cm) at half height, leaf width (cm) at half height, internode length (cm) at half height, internode accounts and panicle length (cm) of 30 randomly selected mature plants were measured and recorded in every site. The morphological variation tendency along salinity grads was analyzed using EXCEL and Statistica software package.

RAPD analysis

1. Sampling and DNA extraction

Young leaves of 10 plants whose space between was wider than 20 meters were separately collected randomly from each population in May. 2000. The materials were rapidly delivered to the lab and stored at 4℃, total DNA extraction was performed using CTAB method (Koppitz 1999) within 7 days. The templates DNA ware suspended in 100μl sterile water and, stored at -20℃ until PCR after concentration measurement.

2. Primers and RAPD-PCR

12 10-base random primers were used in RAPD, of which 6 primers (A01, A05, G06, N02, Q09, Z20) were taken from Kits of Operon Co. and 6 primers (S3, S4, S5, S8, S11, S20) were taken from Kits of Sangon Co., and all of these PCR products were stable and high polymorphic. RAPD reaction system and program were modified according to Koppitz (1999) and Hofstra *et al*. (2000) in 25μl-volume containing 2.5μl 10 × Reaction buffer, 0.2mM dNTP, 2.0mM $MgCl_2$, 10pmol primer, 1.0units of Taq DNA polymerase, 40~80ng Template DNA. All PAPD-PCR reactions were carried out on a BIOMETRA-TGRANDIENT Themocycler. PCR programmed for 40 cycles each of 60s at 94℃, 60s at 35℃, 120s at 72℃, followed by a final termination step of 7min at 72℃ and cooling.

3. Electrophoresis

PCR products were separated in 1.5% agarose gels (EB 1μg/ml) run in 0.5 × TAE buffer (Tris, Boric acid, EDTA, 10mM, pH 8.0) with 3-volt/cm for about 2.5~3h. The band patterns were observed and imaged using Labworks3.0 and saved as BMP file on hard disk.

4. Data analysis

Molecular weight of every band was calculated by DL2000 produced by *TaKaRa* as molecular weight standard. The locus number of every primer was recorded according to band pattern from anode to cathode, every band as one locus. Band positions on the gels were determined visually and the band patterns were transformed into a binary character matrix with 1 for presence or 0 for absence. The dominant frequency at every locus was calculated in every population with Popgen1.32. The relationship between dominant frequency and salinity was analyzed using EXCEL and Statistica.

RESULTS

Morphological variation along salinity

The averages of morphological characters within every population were listed in Table 2. Aanalyzing 7 morphological characters of 15 *P. australis* populations by statistics, it indicated that 7 morphological characters presented the similar tendency to vary along with salinity grads. The Fig. 2 showed how three morphological characters such as basal diameter, height, leaf length and salinity distributed among the 15 populations. Three morphological characters were negatively related to soil salinity and morphological average would decrease with salinity rising. Soil salinity was the highest at HS03 site, and its morphological index was the lowest. It was remarkable that the maximum value of leaf length and height didn't appear in the fresh water habitat, but appeared at BZH which soil was slightly salinized. The morphology *P. australis* at this site was very special in contrast with the populations of the other 14 sites (Zhang *et al*, 2003). Its morphological speciality was beyond the influence of salinity, so this population would be excluded while analyzing the relationship between morphological, genetic variation

Table 2. The averages of morphological characters within populations

Sites	Basal diameter (mm)	Height (m)	Leaf length (cm)	Leaf width (cm)	Internode length (cm)	Internodes number	Panicle length (cm)
HS01	5.25	1.81	27.78	1.89	12.57	18.00	27.29
HS02	5.34	1.61	21.33	1.66	9.26	17.45	22.83
HS03	4.05	0.89	18.41	1.57	6.01	19.60	12.28
HS04	4.51	1.58	25.09	2.14	9.22	14.75	20.82
HS05	4.43	1.60	22.30	1.49	9.82	17.65	22.26
DWL	5.17	1.41	21.23	1.68	8.19	16.65	21.50
KD	5.04	1.44	25.17	1.87	8.88	16.70	23.69
GD	6.19	1.83	31.91	2.29	12.68	21.35	23.82
LZH	5.24	1.54	26.10	1.99	9.03	15.95	24.10
LJ	4.52	1.87	24.68	1.54	12.05	16.35	21.95
BZH	8.86	4.08	42.70	2.96	27.37	28.95	17.59
DLH	5.21	2.28	26.93	1.63	11.97	18.60	19.64
ZHG	6.15	2.85	43.00	3.00	11.82	21.80	30.31
DPH	6.31	2.93	44.09	3.81	9.40	23.15	27.70
NYH	5.95	2.83	45.31	3.62	11.16	21.55	33.54

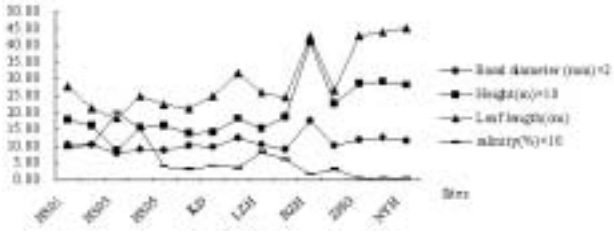

Fig. 2. Basal diameter, height, leaf length and salinity in different sites.

and salinity.

From the analyses on the correlation between morphological characters and salinity among 14 populations except BZH, we could find that all 7 morphological characters were negatively related to salinity (Table 3). It indicated that soil salinity was an important and dominant ecological factor to influence the morphological form in the investigated area.

RAPD variation along salinity

Total 194 loci were amplified in the 15 populations. Most loci did not show remarkable relativity with salinity because RAPD is a neutral marker. Although a few loci were related to salinity, It still gave the primary evidence for genetic basis of ecological adaptation to salinity of *P. australis*.

The distribution of OPA01-10, OPG06-7 and salinity in 15 populations was displayed in Fig. 3. RAPD frequency with salinity didn't show such tendency as morphological characters gradually varying along with salinity. For example, OPA01-10 frequency was high in populations from salinized habitats, and lower in populations from freshwater habitats. While in populations from salinized habi-

Table 3. The correlation tests between averages of morphological characters and salinity among populations

	Basal diameter	Height	Leaf length	Leaf width	Internodes length	Internodes amounts	Panicle length
Correlations	-0.7598	-0.8955	-0.7938	-0.6856	-0.5477	-0.6084	-0.6955
P	p=0.002**	p=0.000**	p=0.001**	p=0.007**	p=0.043*	p=0.021*	p=0.006**
N	14	14	14	14	14	14	14

** p<0.01, Very significant differentiation, * p<0.05, Significant differentiation.

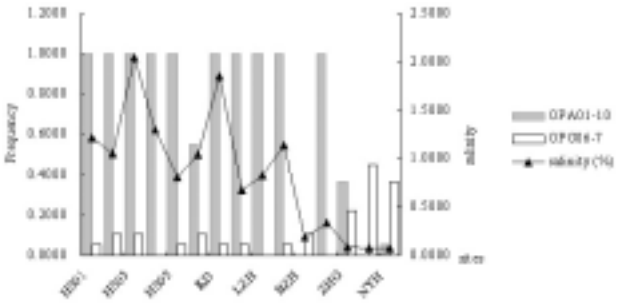

Fig. 3. Frequencies of OPA01-10 and OPG06-7, salinity in different sites.

tats (HS01~DLH), its frequency didn't vary along with the salinity. BZH habitat was slightly salinized, but OPA01-10 band was absence, and this population was genetic special too. The detailed genetic analysis for BZH will be discussed in another paper.

The correlation analysis between RAPD frequencies and salinity among 14 populations except BZH showed that the frequencies of 13 loci were significantly related to salinity (Table 4). Thereinto, 4 loci such as OPA01-10, OPA01-19, SAN05-10, SAN08-4 were significantly positive related to salinity, and showed the preference to salinized habitats, 9 loci such as OPA01-9, OPQ09-11, SAN03-9, SAN11-10, OPA01-22, OPG06-7, OPG06-15, SAN05-3, OPZ20-5 were significantly negative related to salinity, and showed the preference to fresh water habitats. Band patterns of LJ and NYH by OPG06 were showed in Fig. 4. The band OPG06-7 was detected

Table 4. The correlation tests between dominant frequencies at some loci and salinity

Loci	Correlations	p	N
OPA01-10	0.6760	0.008**	14
OPA01-19	0.7028	0.005**	14
SAN05-10	0.7490	0.002**	14
SAN08-4	0.6829	0.007**	14
OPA01-9	-0.6717	0.009**	14
OPA01-22	-0.5592	0.038*	14
OPG06-7	-0.5735	0.032*	14
OPG06-15	-0.5841	0.028*	14
OPQ09-11	-0.6805	0.007**	14
SAN03-9	-0.6780	0.008**	14
SAN05-3	-0.6195	0.018*	14
SAN11-10	-0.6831	0.007**	14
OPZ20-5	-0.5889	0.027*	14

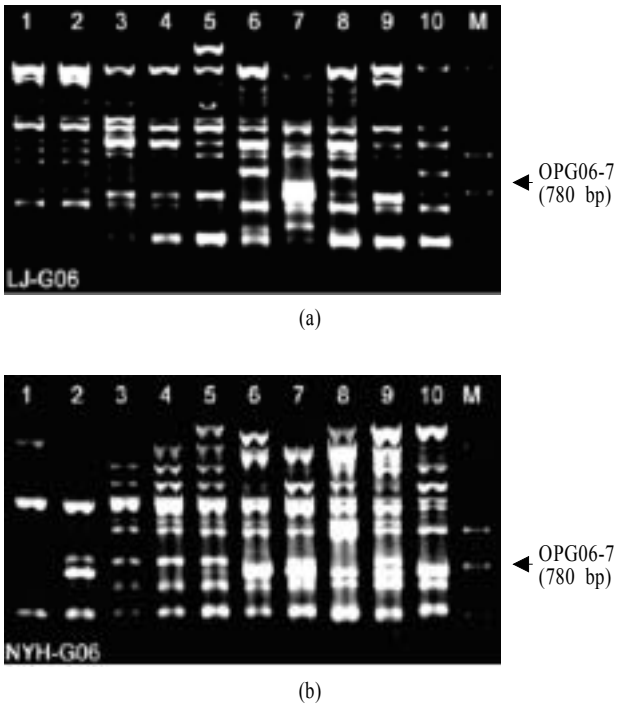

Fig. 4. The RAPD bands pattern of LJ (a) and NYH (b) by OPG06. Samples ranged from lane 1 to 10. Lane M is molecular weight standard (DL2000 produced by *TaKaRa*). Only 1 individual with band OPG06-7 was detected in LJ, but 6 individuals in NYH.

with only one individual in LJ, but with 6 individuals in NYH.

Morphological and RAPD variation

The correlation analysis between 13 loci related to salinity and 7 morphological characters were performed. The results (Table 5) showed most loci were significantly related to morphological characters, but this evidence was not enough to prove whether the RAPD loci were corresponding to morphological characters or not. However, this facilitated us to understand the relation among genetic variation, morphological variation and salinity adaptation.

DISCUSSION

Salinity and morphological variation

The results showed that salinity significantly depressed growth of *P. australis*. Morphological adaptation of *P. australis* to salinity probably came from two aspects. First was high plasticity of morphological character that was controlled only by environment and had no genetic basis. Morphological characters varied continuously along with the environment grads and responded rapidly to the change of environment, so it could be observed in a short term

Table 5. The correlation tests between RAPD loci and Morphological Characters

Loci	Basal diameter	Height	Leaf length	Leaf width	Internodes length	Internodes amounts	Panicle length
OPA01-10	-0.497	-0.427	-0.552*	-0.621*	0.051	-0.221	-0.588*
OPA01-19	-0.663*	-0.773**	-0.866**	-0.904**	-0.0733	-0.637*	-0.702**
SAN05-10	-0.658*	-0.834**	-0.777**	-0.663*	-0.626*	-0.742**	-0.542*
SAN08-4	-0.717**	-0.768**	-0.836**	-0.757**	-0.489	-0.479	-0.783**
OPA01-9	0.659*	0.833**	0.907**	0.935**	0.1591	0.805**	0.682**
OPA01-22	0.483	0.658*	0.728**	0.635*	0.293	0.519	0.686**
OPG06-7	0.615*	0.725**	0.799**	0.883**	-0.016	0.778**	0.592*
OPG06-15	0.396	0.596*	0.529	0.509	-0.059	0.561*	0.225
OPQ09-11	0.791**	0.692**	0.792**	0.797**	0.268	0.823**	0.433
SAN03-9	0.571*	0.770**	0.795**	0.858**	0.0447	0.706**	0.415
SAN05-3	0.649*	0.688**	0.816**	0.822**	0.316	0.706**	0.682**
SAN11-10	0.603*	0.756**	0.739**	0.792**	0.124	0.736**	0.441
OPZ20-5	0.604*	0.748**	0.783**	0.859**	0.049	0.740**	0.502

such as one month, even within one week. The second was the morphological variation related to genetic variation. The morphological variation within this scope had genetic basis, which was the accumulated results of genetic and morphological characters adapting to environment within a long term. In this situation, the morphological variation along with environment grads probably showed discontinuity. Morphological characters responded slowly to the change of environment in this scope, probably could be observed after a lot of years. The 14 populations except BZH were separated into two morphological types, saltwater reed and freshwater reed. The morphological difference between different types were significant, however, the morphological difference between different populations belonging to one type were not significant (Zhang *et al.* 2003). Thus, the significant morphological difference between different types was probably related to ecological genetic adaptation, but the continuous morphological variation among populations belonging to one type probably formed mainly under plasticization of ecological factors.

Salinity and genetic variation

RAPD only detected 14 loci that were significantly related to salinity in this study, but nothing was known about whether or how these loci participated in the regulation of genetic adaptation to salinity. In order to discuss mechanism of genetic adaptation of *P. australis* to salinity, the gene related to salinity adaptation should be known, and the physiological and morphological adaptation process should be fully understood too. Nowadays many kinds of molecular markers such as RFLP, VNTR, RAPD, SSR and AFLP have been applied in population genetics. Most molecular markers are neutral and limited to explore ecological genetic adaptation under the conditions that nothing was known about genome and the function of gene of *P. australis*. It is an approach to get the DNA segments related to ecological factors from genome by RAPD, and then perform detailed research on these segments. But if the segment was not gene with integrated function, the following study couldn't go on. The detailed studies on phenotype of *P. australis* and salinity adaptation has been performed (Zhang *et al.* 2003, Zhao *et al.* 1998), but it is very difficult to connect genetic, physiological and morphological response to ecological factors such as salinity.

Morphological and genetic variation

All of the characters used in this study are quantitive traits and its genetic regulation very complex (Hedrick 1999). Although this study suggested that morphological characters showed significant relativity to some RAPD loci, it was not enough to prove whether there were corresponding relations between these RAPD loci and morphological character. Although the evidences were not sufficient and not straight, genetic influences on morphological characters of *P. australis* were no doubt decisive. There were also some reports about special clone speciality of *P. australis* in morphology and genetics. The influence of genetic variation on morphological variation could be considered from two aspects. First was random genetic polymorphism that probably led to morphological polymorphism. It often showed neutral to ecological factors, for example, clonal

diversity and the morphological difference within population (Zhang *et al*. 2003). Second was genetic polymorphism with ecological adaptability. It may be led to morphological polymorphism with ecological adaptability. The mechanism genetic polymorphism with ecological adaptation was in argument, probably being formed gradually under the pressure of natural selection. Nowadays, it still was difficult to fully understand the influence of genetic variation on morphological characters.

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