POLISH POLAR RESEARCH
vol. 32, no. 1, pp. 15–26, 2011 doi: 10.2478/v10183-011-0005-9

Genetic and epigenetic studies on populations of *Deschampsia antarctica* Desv. from contrasting environments on King George Island

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Abstract: Populations of Antarctic hairgrass *Deschampsia antarctica* Desv. from King George Island exhibit variation in many traits. The reason for that is not evident and could be addressed to variable environmental conditions. Obviously, phenotypic variation could be due to stable or temporal changes in expression pattern as the result of adaptation. Stable changes could be due to mutations or site DNA methylation variation that modified expression pattern. Recently, metAFLP approach was proposed to study such effects. A variant of methylation sensitive AFLP (Amplified Fragment Length Polymorphism), based on the isoschizomeric combinations *Acc65*I/*Mse*I and *Kpn*I/*Mse*I was applied to analyze the sequence and site DNA methylation differences between two *D. antarctica* populations exhibiting morphological dissimilarities. Both DNA sequence mutations and site methylation pattern alternations were detected among and within analyzed populations. It is assumed that such changes might have originated as the response to environmental conditions that induced site methylation alternations leading to phenotypic variation of *D. antarctica* populations from South Shetland Islands.

Key words: Antarctic, South Shetland Islands, *Deschampsia antarctica*, genetic/methylation diversity.

Introduction

Vascular plants have an intricate mechanisms enabling them to response to environmental changes and numerous biotic and abiotic stresses (Bruce *et al.* 2007) sometimes leading to variable but stable phenotypes among the offspring of a common ancestor. Such dissimilarities might be due to mutations (Rout *et al.* 2006) or changes in DNA methylation (Chinnusamy and Zhu 2009). Although

mutations are rare and, if affecting plant functioning are eliminated during successive reproduction cycles (Rout *et al.* 2006), changes in DNA methylation may influence gene expression patterns modifying morphological traits (Wahl *et al.* 2001). Methylation changes might be either temporal (Finnegan 2001) allowing for a certain plasticity of gene expression (Grant-Downton and Dickinson 2005) and are usually easily reversible (Grant-Downton and Dickinson 2006) or inherited via successive generations (Kaeppler *et al.* 2000). Modifications of the DNA may play a crucial role in plant adaptation since even plants with a very low genetic diversity may easily adopt to a wide range of growing conditions (Lukens and Zhan 2007).

Antarctic hair grass (*Deschampsia antarctica* Desv.) is a good example of a species, which demonstrates low diversity in their whole range (Holdereggere*t al.* 2003; Chwedorzewska and Bednarek 2008; van de Wouw *et al.* 2008). It is a tussock grass that thrives in the harsh conditions of the Maritime Antarctic. Populations of *D. antarctica* in the Antarctic occur on coastal areas of Antarctic Peninsula and on associated archipelagos, often separated from one another by natural barriers limiting gene flow (Smith 2001). *D. antarctica* displays wide ecotypic variation, colonizing diverse habitats ranging from mineral to organic soils, from extremely dry to occasionally inundated by sea water (Smith 2003), and from nutrient-deficient sites to habitats enriched in nutrients by animals and sea spray (Smith 2003; Nędzarek and Chwedorzewska 2004). Populations from such sites differ in morphological and anatomical traits (Corner 1971; Giełwanowska 2003 a, b; Chwedorzewska *et al.* 2004, 2008). Although phenotypic dissimilarities of *D. antarctica* among sites are very common (Corner 1971; Giełwanowska 2003 a, b) the origin of such variation is not clear. Microclimate, including factors such as moisture availability, temperature, ground-level wind speed or snow cover, has a major influence on plant growth (Alberdi *et al.* 2001) and may modify morphological traits. However, little is known about the effect of nutrients on the performance of Antarctic vegetation (Kappen 2000) and how they may induce phenotypic variation among *D. antarctica* localities. The AFLP technique has been adapted for the analysis of cytosine methylation in plants (Bednarek *et al.* 2007) based on the use of isoschizomers that show differential sensitivity to cytosine methylation. *Acc65*I and *Kpn*I are isoschizomers, which differ in their sensitivity to template methylation. Both enzymes recognize the: 5'… GGTAC^C… 3' sites and cut unmethylated DNA. The former is insensitive to *dam* methylation, but its activity is blocked by both *dam* and CpG methylation, while the latter is insensitive to all forms of methylation. Both enzymes were combined with the methylation-insensitive *Mse*I.

The main goal of the study was to verify whether two *D. antarctica* populations from South Shetland Islands exhibiting distinct phenotypes and growing under diverse soil and moisture conditions differ at the DNA sequence and/or site DNA methylation pattern using methylation sensitive AFLP approach (Bednarek *et al.* 2007).

Materials and methods

Samples of *Deschampsia antarctica* were collected during the austral summer season of 2007/2008 on the western shore of the Admiralty Bay in the vicinity of Polish *Arctowski* Station (King George Island, South Shetland Islands, 62°09'S, 58°28'W) in the area of ASPA (Antarctic Specially Protected Area) 128. Special care was undertaken to collect plant material at a similar stage of development. Samples of *D. antarctica* were obtained from two localities that differ remarkably in ecological conditions (Chwedorzewska *et al.* 2004; Nędzarek and Chwedorzewska 2004). Samples from the first one (population A) grew far from animal colonies on extremely dried, exposed to wind mineral soils with strong nutrient-deficiency (Nędzarek and Chwedorzewska 2004). Plants were small, olive-green and formed small single flat individual tufts. The samples from the second location (population B) occupied waterlogged seepage areas occasionally inundated by water, highly nutrient-enriched, notably with ammonium and nitrate, located very close to penguin rookery (Nędzarek and Chwedorzewska 2004). *D. antarctica* grew in uninterrupted patches forming extensively closed swards that covered even several square meters of ground. Animal feces were in direct contact with plants that were green, robust, had well-developed foliage and erect culms (Chwedorzewska *et al.* 2004).

DNA isolation. — Fifty fresh shoots from each population were collected, then washed in 70% ethanol and dried on a Wattman paper. DNA was extracted from about 50 mg of fresh tissue following recommendations of the manufacturer of the DNA extraction kit (NucleoSpin®Plant, Macherzy-Nagel). DNA samples were kept at -20°C during transportation.

Table 1

Adapter and primer sequences; NN – any combination of the nucleotides at the primers 3'ends

Adapter/Primer	Sequence (5'→ 3')
Adapters *Acc*65I	CTC GTA GCA TGC GTA CA
	GTA CTG TAC GCA TGC TAC
Adapters *Kpn*I	CTC GTA GCA TGC GTA CAG TAC
	ACT CGT ACG ACT GT
Adapters *Mse*I	TAC TCA GGA CTC ATA
	GAG TCC TGA GTA GCA G
*Acc*65I/*Kpn*I pre-selective primer	GAT GAG TCC TGA GTA AC
*Mse*I preselective primer	GAT GAG TCC TGA GTA AC
*Acc*65I/*Kpn*I – ANN – selective primer	CAT GCG TAC AGT ACC xxx
*Mse*I – CNN – selective primer	GAT GAG TCC TGA GTA ACxx

AFLP analysis. — The AFLP procedure followed a standard AFLP method (Vos *et al.* 1995) with modifications (Bednarek *et al.* 2007). DNA sample (500 ng) was cut with *Acc*65I/*Mse*I, while the other one (500 ng) with *Kpn*I/*Mse*I enzymes following adaptor ligation, pre-selective amplification and selective amplification steps. The selective amplification was performed in the presence of ^{32}P 5' labelled selective primer. Five selective primer combinations (Table 1) were applied to all samples. The PCR products were separated on 5% PAGE and exposed to X-ray films (Bednarek *et al.* 2007). Two independent repeats of amplification and PAGE separations were performed.

Data analysis and statistics. — Reproducible, clearly distinguishable, AFLP fragments were scored in a form of a binary matrix with presence (1) or absence of a band (0) (Fig. 1). Not-redundant markers were searched for using AFLPop 1.1. software (Duchesne and Bernatchez 2002). Principal Coordinate Analysis (PCoA) was performed with Xlstat v.7.5.2 excel add-in software (http://www.xlstat.com, Addinsoft) to visualize differences between populations under study. Analysis of population structure was performed in STRUCTURE 2.0 software with default parameters suggested in the manual (Pritchard and Wen 2003).

GenAlex 5.3 software (Peakall and Smouse 2001) was used to evaluate allele frequencies; number of markers shared among individuals with a frequency

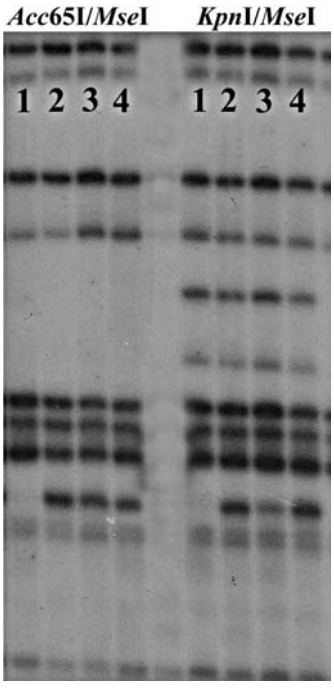

Fig. 1. Fragment of gel electrophoresis of AFLP products for four individuals from two populations for CpXpG-TGC/CGG primer pair combination.

greater or equal to 5%; number of private markers; Shannon's Information Index (I); Expected Heterozygosity (He); Unbiased Expected Heterozygosity (UHe); percentage of polymorphic alleles (P%); Nei's and Unbiased Nei genetic distance and analysis of molecular variance (Φ_{PT}) (Shannon and Weaver 1949; Nei 1978; Halliburton 2004; Hammer *et al.* 2001). Polymorphic Information Content (PIC) was evaluated according to the following formula PIC = Σ 0.25*(1-f_i)*f_i, where f_i is the frequency of the ith allele.

Neighbour Joining clustering was constructed on the basis of the data from all sampled individuals with PAST using Jaccard genetic distances. The robustness of the branches was estimated using 1000 bootstrap replicates. Gene flow rate was calculated using the equation $N_m = 0.5\ (1- \Phi_{PT})/\Phi_{PT}$.

Results

In total 190 and 193 AFLP fragments amplified by five primer combinations in *Acc65*I/*Mse*I and *Kpn*I/*Mse*I platforms were identified, respectively; 100 (53%) and 93 (48%) bands were polymorphic, while five of them were unique for *Acc65*I/*Mse*I and eight for *Kpn*I/*Mse*I digests (Table 2).The number of markers was reduced after elimination of redundancy. Finally, there were 83 and 80 markers amplified among individuals from A and B using *Acc65*I/*Mse*I AFLP platform. *Kpn*I/*Mse*I based profiling resulted in 70 (A) and 70 (B) AFLPs. Most of the bands were present with a frequency higher than 5% within the given pop-

Table 2

Arrangement of data generated with the selected primer pairs for both restricted enzymes combinations

Enzyme combination	Primers	Detected bands (in total)	Polymorphic bands	Polymorphic bands (%)	Number of unique bands
*Acc65*I/*Mse*I	CpXpG-TGC/M-CGG	40	25	63	1
*Kpn*I/*Mse*I	CpXpG-TGC/M-CGG	39	19	49	0
*Acc65*I/*Mse*I	CpG-GGC/M-CAA	48	28	58	0
*Kpn*I/*Mse*I	CpG-GGC/M-CAA	51	34	67	3
*Acc65*I/*Mse*I	CpXpG-AGG/M-CAG	28	17	61	0
*Kpn*I/*Mse*I	CpXpG-AGG/M-CAG	32	14	44	4
*Acc65*I/*Mse*I	CpXpG-AGA/M-CCC	26	14	54	0
*Kpn*I/*Mse*I	CpXpG-AGA/M-CCC	26	12	46	0
*Acc65*I/*Mse*I	CpXpG-AGC/M-CCA	48	16	33	4
*Kpn*I/*Mse*I	CpXpG-AGC/M-CCA	45	14	31	1
*Acc65*I/*Mse*I	Total	190	100	53	5
*Kpn*I/*Mse*I	Total	193	93	48	8

Table 3

Arrangements of population genetic characteristics for A and B. N – number of individuals, P% – percentage of polymorphic alleles, within population, I – Shannon's Information Index; PIC – Polymorphism Information Content, He – Expected Heterozygosity; UHe – Unbiased Expected Heterozygosity

Population	N	P %	I	PIC	He	UHe
*Acc*65I/*Mse*I						
A	24	80.81	0.231±0.020	0.023	0.135±0.014	0.138±0.015
B	24	78.79	0.220±0.021	0.021	0.129±0.015	0.132±0.015
Mean		79.80±1.01	0.225±0.015	0.022	0.132±0.010	0.135±0.011
*Kpn*I/*Mse*I						
A	24	86.08	0.258±0.023	0.026	0.152±0.016	0.155±0.017
B	24	84.81	0.247±0.022	0.025	0.144±0.016	0.147±0.016
Mean		85.44±0.63	0.252±0.016	0.025	0.148±0.011	0.151±0.011

Table 4

Matrix of Genetic distance between A and B populations and gene flow (N_m) value

AFLP platform	Nei GD	Nei's UGD	Φ_{PT}	N_m
*Acc65*I/*Mse*I	0.010	0.006	0.047 (p = 0.001)	10.12
*Kpn*I/*Mse*I	0.018	0.015	0.031 (p = 0.002)	15.63

ulation (A = 58 and B = 57 for *Acc65*I/*Mse*I; A = 51 and B = 57 for *Kpn*I/*Mse*I). The populations from A amplified 16 (10), while the one from B19 (10) private bands based on *Acc65*I/*Mse*I (*Kpn*I/*Mse*I) AFLPs. The markers shared among individuals of both populations were highly polymorphic for *Acc65*I/*Mse*I (A = 80.8, B = 78.8) and *Kpn*I/*Mse*I (A = 86.1, B = 84.8). Shannon's Information Index were 0.231 (A) and 0.220 (B) for the *Acc65*I/*Mse*I platform and were 0.258 (A) and 0.247 (B) for *Kpn*I/*Mse*I. According to this parameter the available markers were informative enough to carry on further analysis ($I \geq 0.3$). Population A was more heterozygous than the B one for both platforms (Table 3). Analysis of molecular variance of *D. antarctica* samples revealed that 5% (Φ_{PT} = 0.047, p = 0.001) and 3% (Φ_{PT} = 0.031, p = 0.002) (Table 4) of variance were due to intra-population differences in *Acc*65I/*Mse*I and *Kpn*I/*Mse*I platforms, respectively. So, both populations were closely related to each other, however higher variation revealed *Acc*65I/*Mse*I (Table 4). A low genetic distance between both populations and lack of population structuring were recorded. Gene flow (*Nm*) value between the two populations was estimated to be 10.12 or 15.63 depending on AFLP platform (Table 4).

The PCo Aanalyses performed based on *Acc*65I/*Mse*I (Fig. 2) and *Kpn*I/*Mse*I (Fig. 3) AFLPs demonstrated that while some samples originating from both locations occupied central part of the cloud, the others evidently moved to its opposite

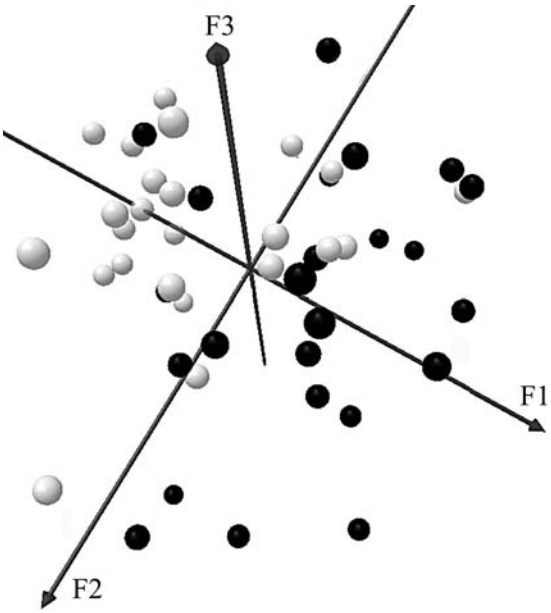

Fig. 2. Principle Coordinate Analysis based on *Acc*65I/*Mse*I AFLP platform. Principle Coordinates explain 9.767, 5.979 and 5.737 percentage of variability (21.483% cumulative); black dots – individuals from population A, gray dots – individuals from population B.

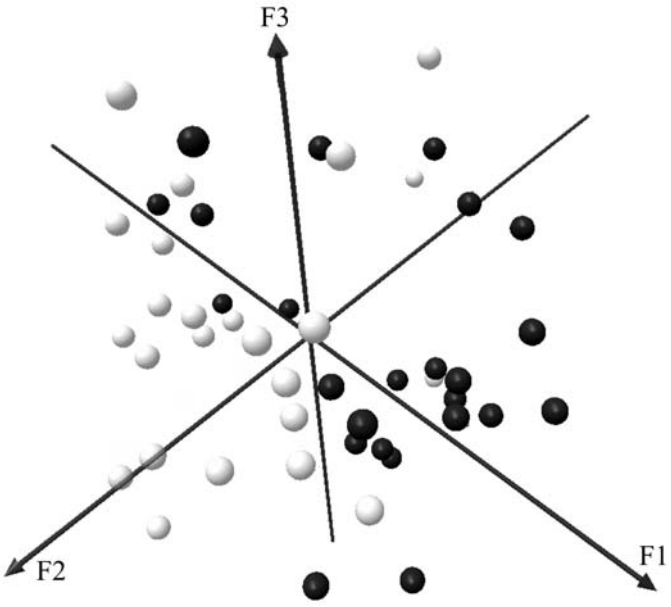

Fig. 3. Principle Coordinate Analysis based on *Kpn*I/*Mse*I AFLP platform. Principle Coordinates explain 8.229, 7.287 and 6.457 percentage of variability (21.974% cumulative); black dots – individuals from population A, gray dots – individuals from population B.

parts following location clue. Such a grouping is even more evident if the *Acc*65I/*Mse*I AFLPs are considered (Fig. 3). In both cases the differences were significant ($\alpha = 0.01$). Neighbour Joining clustering show very similar results (data not shown).

Discussion

Previous studies using *Eco*RI/*Mse*I AFLP platform showed that *Deschampsia antarctica* populations exhibited very low genetic variability even if they were spatially separated by distance (Holderegger *et al.* 2003; Chwedorzewska and Bednarek 2008; van de Wouw *et al.* 2008). The genetic structure of populations that are in close vicinity are nearly identical even if they exhibit evident morphological and anatomical differences (Chwedorzewska *et al.* 2004; Chwedorzewska *et al.* 2008). It is well established that *Eco*RI/*Mse*I AFLPs, at least in case of another species from *Poaceae*, map to centromeric and telomeric regions forming tightly linked groups of markers and are not evenly distributed along chromosomes (Bednarek *et al.* 2003). Moreover, *Eco*RI/*Mse*I platform applied to double haploids of barley failed to identify any variation (Oleszczuk *et al.* 2002) while other AFLP platforms did (Bednarek unpublished data). Thus, such a distribution of markers, if this is a case in *D. antarctica*, may lead to underestimation of genetic variation what makes the choice of AFLP platform crucial. Alternatively, phenotypic dissimilarities could be addressed to modifications that are fixed on DNA methylation level and influence expression pattern. According to Cubas *et al.* (1999) differences in many plant traits caused by methylation can occur at a high frequency within natural populations generating phenotypic variation that is not correlated with genetic one.

The site DNA methylation variation could be induced by environmental stimuli such as i.e. water stress that can cause change in cytosine methylation throughout the genome (Labra *et al.* 2002). Several recent reports have shown that numerous environmental stresses such as cold (Stewards *et al.* 2002), oxidative (Cassells and Curry 2000), osmotic stress (Kovařik *et al.* 1997) often in combination with ornitocoprophily, lead to altered DNA methylation status (Madlung and Comai 2004).

In those studies we have argued that phenotypic variation among analyzed *D. antarctica* populations (Chwedorzewska *et al.* 2004) might be the result of the environmental conditions, namely soil, moisture and exposition rather than genetic differences. To verify whether those conditions might be responsible for phenotypic variation between the populations metAFLP approach was involved (Bednarek *et al.* 2007). Since *Acc*65I and *Kpn*I are isoschizomers, which differ in methylation sensitivity, it should be easy to distinguish genetic and epigenetic changes by comparing the populations based on both platforms. Based on PCoA

both populations were placed at opposite poles of the graph. Whereas methylation sensitive platform revealed that some individuals were mixed for both AFLP platforms. The data presented in this paper clearly demonstrate that the proper choice of the AFLP platform is very important especially in case of species with narrow germplazm. Evidently the populations differ both at the sequence and site DNA methylation levels. Assuming that analysis of molecular variance demonstrate that 5% of variance is due to sequence and methylation variation and 3% to sequence, roughly 2% of variance is due to methylation pattern.

Evidently, sequence changes overwhelm methylation, despite of the very high gene flow between populations. It is difficult to conclude whether sequence variation or methylation itself is responsible for phenotypic dissimilarities and additional studies are needed to address this question. Nevertheless, even small differences in methylation polymorphisms detected among analysed populations may point to plastic response to stress and may be important in acclimation to a range of environmental conditions. Thus, populations in consistently different sites might differentiate into local ecotypes (Heschel *et al.* 2004). Moreover, deleterious sequence changes are eliminated during successive generative cycles, thus those detected by us either appear within non-coding regions or have positive effect on the populations and reflect adaptation processes induced by mutation mechanism. It might be expected that sequence variation within both populations should differ, if microclimate condition at least in one of the locations favour such events. PCoA performed based on *Kpn*I/*Mse*I platform did not show dissimilarities in distribution of representatives within each population what could be interpreted in terms of random nature of mutations and supporting notion that methylation play the clue role in phenotypic variation among the populations.

Plant communities of Antarctic environments are expected to be very sensitive to changes in climate or consequential processes (Frenot *et al.* 2005). Botanical responses to recent climate amelioration are already visible in the Maritime Antarctic and Subantarctic, in the form of rapid growth of local population density and distribution of the two Antarctic vascular plant species (*Colobanthus quitensis* and *Deschampsia antarctica*).This changed their reproductive patterns with a greater incidence of successful sexual reproduction and increased seed output (Fowbert and Smith 1994). So, probably high capability to adaptation to very wide range of environmental conditions can buffer urgent climate changes. However, colonization of Maritime Antarctic by alien species (Chwedorzewska 2008) changing in patterns and importance of competition, will itself be one of the major outcomes of climate change for native Antarctic species.

Acknowledgements. — We would like to thank two anonymous reviewers for constructive advice that has improved our paper. This research was supported by grant IPY/27/2007.

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Received 9 February 2010
Accepted 14 February 2011