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Vukosavljev Mirjana, Di Guardo M, van de Weg E, Arens P, Smulders MJM

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Index

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Miscellaneous

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The genetic dynamics and recent colonization of Southeastern Brazil by stingrays (Chondrichthyes: Potamotrygonidae).

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Summary

Among the organisms that comprise the Elasmobranchii group, the rays of Potamotrygonidae family include 16 to 20 species distributed into the genera *Paratrygon*, *Plesiotrygon*, *Potamotrygon* and *Heliotrygon*, which are found in the major river systems of South America. Molecular genetic markers were used to study the processes of dispersion and colonization of specie of freshwater stingray, *Potamotrygon motoro* in the upper Parana River basin in Southeastern Brazil in the recent time, after the construction of Itaipu dam. The population analysis for *P. motoro* using D-loop sequences indicated a very low level of intraspecific diversity. It is hypothesized that the low values of genetic differentiation found for these molecular genetic markers among the studied populations is possibly due to the fact that, besides the extension of the migration for new colonized areas they are still in process of genetic divergence.

Introduction

The Potamotrygonidae family comprises about 20 species of Elasmobranchi inhabiting freshwater environments and included in the genera *Plesiotrygon*, *Paratrygon*, *Potamotrygon* and *Heliotrygon*. These species are restricted to major river systems in South America, including rivers of Venezuela, Guiana, Suriname, French Guiana, Colombia, Peru, Bolivia, Paraguay, Argentina, Uruguay and Brazil (Achenbach & Achenbach 1976, Rosa 1985). In the latter, the largest number of species is found in the Amazon Basin (about 13 species), except for the species under description process and/or not yet described (Charvet-Almeida et al. 2002). In the central western and southeast regions, Paraguay-Paraná Basin is the home to around seven nominal

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Figure 1 - The South America map, highlighted the collection points of the Paraná Basin. FZ – Foz do Iguaçu, PR – Porto Rico, IS – Ilha Solteira.

species. However, this number may vary due to possible existence of synonyms and species not yet described (Rosa 1985, Carvalho et al. 2003). Until the late 1970s, stingrays were noted only in sections downstream to "Sete Quedas" waterfalls in Paraná River, a major geographical barrier located near the city of Guaíra, Paraná State (Vilela et al. in 2004).

This natural barrier that isolated for a long period most fish fauna components of the Upper Paraná River from the remnant fauna of downstream systems, ceased to exist due to the construction of Itaipu Hydroelectric Dam about 70 kilometers downstream Sete Quedas, with the reservoir filled in 1982. This fact enabled new species to colonize the site located upstream to Guaíra and in this new and large environment, stingrays (*Potamotrygon motoro*, *Potamotrygon falkneri* e *Potamotrygon* sp.) and other local fish species started to play the role of invasive species, causing impacts of different dimensions on the local native aquatic fauna (Brazil 1998, Vilela et al. 2004), which still needs to be further studied and measured.

Considering the relative lack of molecular data for representatives of the family

Potamotrygonidae, this paper address basic questions related to molecular characterization of species belonging to this group, identifying the genetic diversity of populations and providing informations to better understanding the dynamics of the colonization process established by these species in the occupation of new environments.

Methods and Material

Surveys carried out in three sites along the Paraná River in Foz do Iguaçu (FI), Porto Rico (PR), and Ilha Solteira (IS) (Figure 1), resulted in 60 individuals of the species *Potamotrygon motoro*. Four individuals in this sample showed distinct color pattern, receiving the name of *Potamotrygon* sp. Another specie *P. falkneri* was also collected in Ilha Solteira (4 individuals), were included in the analyses to calculate genetic divergence between species.

All individuals were identified and tissue samples were stored in 95% ethanol for molecular studies. DNA was extracted from tissue samples using a standard proteinase K and phenol/chloroform protocol (Sambrook et al. 1989). Molecular identification of individuals was performed using a short segment of 648 nucleotides of 5 'end of the mitochondrial cytochrome oxidase I gene (COI I) that would be sufficient to identify them at species level (Hebert et al. 2003a, 2003b). The control region of mitochondrial DNA (D-loop) was also amplified for population studies. Each PCR reaction mixture contained approximately 30 ng of genomic DNA, 10x buffer (100 mM of Tris–HCl, 500 mM of KCl, 15 mM of MgCl2), 0.3 mM of dNTP (7.5µl), 0.3 mM of each primer (1.5µl), 3 mM of MgCl2 (1.5µl), 1U of Taq Polymerase(1µl) and ddH2O (8.5µl) for a final volume of 25 µl. Cycling times were as follows: 5 min at 94 °C; 35 cycles of 1 min at 95 °C (denaturation), 30 sec at 55 °C /57°C (annealing) and 45 sec at 72 °C (elongation); and a final extension step for 5 min at 72 °C was used. A negative control was also included to check for contamination.

Distance-based trees were obtained and drawn with the program MEGA (Kumar et al. 2008) on the same set of sequences. The Kimura's 2-parameters distance measure was used, which also accounts for different transition/transversion ratios. Population structure and genetic variance were analyzed using Arlequin 3.01 (Schneider et al. 2000). Genetic diversity was analyzed by estimating haplotype diversity (h) and nucleotide diversity (π) using the methods of Tajima (1983) and Nei (1987). The overall genetic differentiation was tested using the pairwise fixation index (F_{st}) between populations.

Results

Cytochrome oxidase I (COI) - The analyzes of the COI region of the species *P. motoro, P. falkneri* and *Potamotrygon* sp. showed that the average genetic divergence found within populations was 0.3% for *P. motoro* and 0.2% for *P. falkneri* (Figure 2). Comparison between the species *P. motoro* and *Potamotrygon* sp. indicated a genetic distance of 0.3%, corresponding to the same divergence found among *P. motoro* populations, suggesting that the analyzed individuals could belong, in fact, to one same species, *P. motoro*.

Control region (D-loop) - DNA fragments corresponding to the control region (D-loop) of 60 *P. motoro* specimens were amplified and sequenced. The amplification resulted in a fragment of approximately 800bp, and after alignment, 618bp sequen-

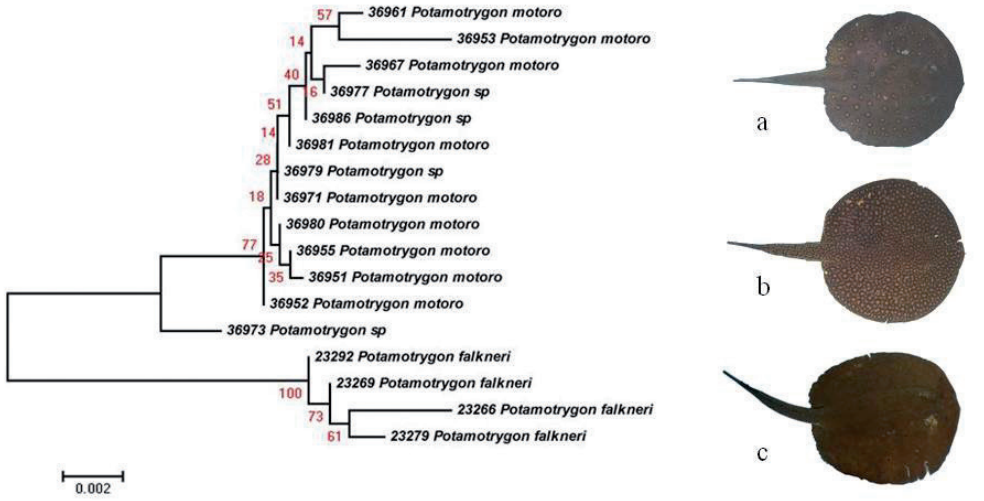

Figure 2 - Dendogram obtained by Neighbour-joining (MEGA 4.0) from COI gene sequences of stingrays specimens from Paraná Basin. Bootstrap values are represented in the respective nodes. a) Potamotrygon motoro, b) Potamotrygon falkneri, c) Potamotrygon sp.

Localidade	*n*	*hn*	*h*	*s*	*π*	*k*
Foz do Iguaçu - PR	25	10	1,00000	12	0,00480	2,96000
Porto Rico – PR	15	5	0,59480	6	0,00104	1,02154
Ilha Solteira - SP	20	6	0,64333	7	0,00486	3,00000

Table 1 – Locality information of analyzed Potamotrygon motoro populations. Number of individuals (n), the number of haplotypes (hn), the haplotype diversity (h), the number of polymorphic sites (s), nucleotide diversity (ϖ) and mean difference pairwise nucleotide (k).

ces were obtained. The average nucleotide composition was 29.0% Adenine, 19.4% Cytosine, 18.7% Guanine and 32.4% Thymine. The transition / transversion rate was 2.1. The population with the highest haplotype diversity was that from Foz do Iguaçu, whereas the populations from Porto Rico and Ilha Solteira showed the lowest haplotype diversity. The nucleotide diversity and the average of pairwise nucleotide differences followed the same pattern (Table 1). The results of *Fst* showed that *P. motoro* populations are little organized, showing significant values between Foz do Iguaçu and Ilha Solteira (0.083).

Discussion

The nucleotide composition of COI gene showed significant punctual mutations among the studied species, similar to those found by other authors (Toffoli 2006,

Manniglia 2010, Pereira 2011). Mitochondrial DNA has a high nucleotide substitution rate, about 5-10-fold higher than that of nuclear DNA (Brown et al. 1971, Harrison, 1989). In the present study, the rates of divergence among species were low compared to the results obtained for other fish species (Pereira 2011), but the few studies carried out with river stingrays indicate low genetic variability among Potamotrygonidae members, especially for populations found in the Paraná River Basin (Maniglia 2010, Pereira 2011). When COI sequences were analyzed using the Neighbour-joining method, four individuals previously identified by as *Potamotrygon sp.* according to their distinct color pattern, showed COI sequence characteristic of *P. motoro*. The variation in color has been found in other species stingrays, as in *P. orbignyi* and *P. scobina* (Toffoli 2006), may be related to adaptations to the environments in which they occur (Allendorf & Luikart 2006).

There are evidences that mtDNA of Elasmobranchii varies at a lower rate than that found in mammals (Martin 1999, Martin et al. 2002) and studies using the D-loop control region proved to be very efficient in population analyzes for marine sharks and rays (Heist 2004, Mendonça 2010). However, only a few studies have been conducted so far with stingrays (Maniglia 2010), including the analysis of *Potamotrygon* populations through the D-loop control region, whose comparisons presented a low genetic divergence among populations. The data obtained revealed that the population from Foz do Iguaçu shares only a few haplotypes with the population from Ilha Solteira, differently from other population (Porto Rico). The sharing of haplotypes for different populations suggests a lack of genetic structuring among them, corroborating the studies of Maniglia, 2010.

The low values found for *Fst* indicated a low organization level among populations of Paraná Basin, may be due to several factors, acting independently or together. The species of stingrays of the upper Paraná River, are not native to this region and do not know the original genetic diversity of populations, these populations may also exhibit low diversity. Another factor to consider is that if the original population has high diversity, the new established population is likely to have lower genetic diversity than the population that originated it. In this case, events resulting from processes of genetic drift, founder effect and / or bottleneck in the process of invasion (Sakai et al. 2001) could be acting on populations in a dynamic way and still undefined.

Conclusion

Since the species of stingrays found in the upper Paraná River are not native to this region, the genetic diversity of the original populations found in individuals of the low part of the river system influenced directly in the organization of the migrating populations. It can be considered that the populations currently existing in this newly occupied environment represent part or the total diversity found in the species. The diversity found among populations can be related to time of divergence and speed of the mixing process in the newly established populations. In this case, events resulting from processes of genetic drift, founder effect and / or bottleneck during the invasion process could, according to Sakai et al. (2001), be acting on populations in a dynamic and still undefined way.

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Inter-populations diversity in Onosma microcarpa (Boraginaceae): Morphological and molecular (ISSR) approach

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Summary

Seventeen populations of *Onosma microcarpa* from 9 different provinces of Iran were investigated for morphological and ISSR markers diversity. These plants grow from north to south across 1200 km and from west to east across 860 km area in mountain slopes and road sides having different climates. Clustering of morphological data separated the populations of Marivan (Kurdestan), Avaj (Hamedan), Dizin (Tehran) and Nikpey (Zanjan) due to their higher degree of morphological differences. Marivan and Avaj populations had significantly higher values fo morphological features and also showed affinity in ISSR characteristics. Therefore, they me be considered as new ecotype in this species. The populations of Belghias (Zanjan) and Taleghan (Ghazvin), Touchal (Tehran), Kolakchal (Tehran), Arak (Markazi), Paveh (Kermanshah), show similarity in morphological characters and are placed in a single major cluster, in which Kolakchal (Tehran) and Arak (Markazi) populations show high affinity. These populations do not form a cline in their geographical distribution. Out of 10 ISSR primers used (alone and in combination), 6 primers produced 67 polymorphic reproducible bands. Some common bands were observed in all populations studied while some bands occurred only in one population and are specific bands. Some ISSR bands were present in all populations except one. All these molecular markers may be used to differentiate the populations studied. Clustering of the populations based on ISSR data also grouped the populations irrespective to their geography. AMOVA test performed among cluster groups obtained showed a significant molecular difference

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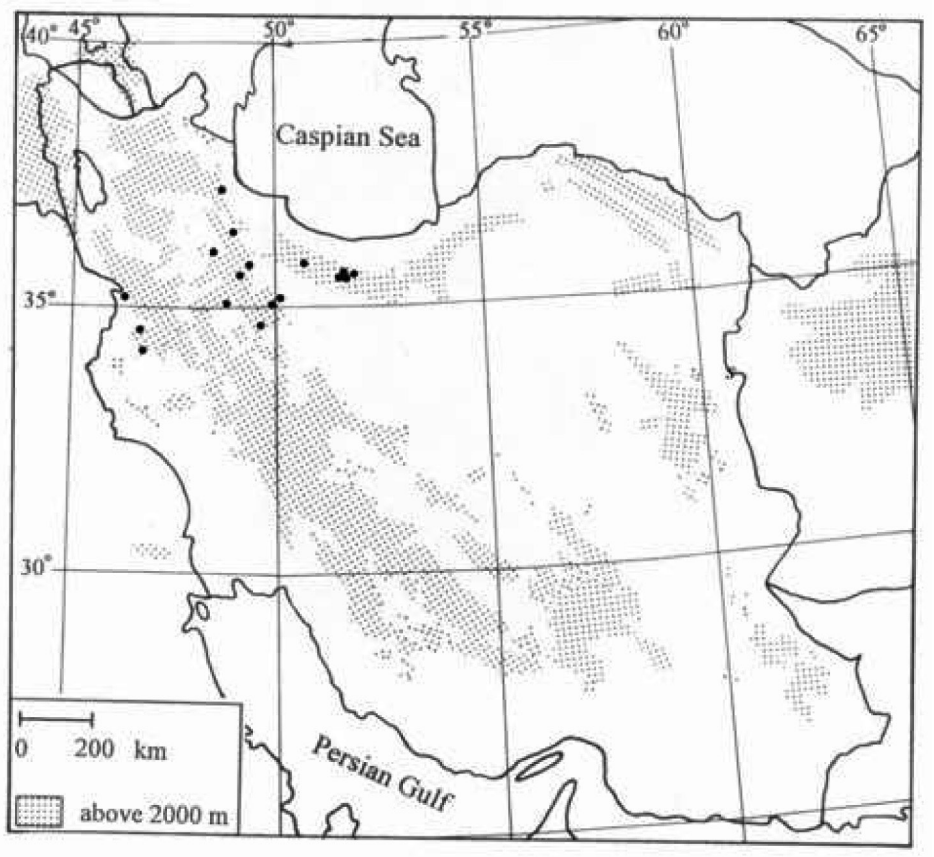

Fig. 1. Distribution map of O. microcarpa populations studied.

among them. However, when similar test performed among populations of different provinces studied showed no significant difference.

Introduction

Onosma is a genus with about 150 species occurring in dry, cliffy and sunny habitats, distributed mainly in Eurasia and Mediterranean regions, having its center of distribution and maximum concentration of species in Iran (Ball 1972, Willis 1973). The genus *Onosma* contains biennale or perennial, hispid herbs, with flowers in terminal cymes, calyx accrescent, stamens inserted at the middle of the corolla and generally 4 nutlets flat at the base (Binzet *et al*. 2010). It contains about 60 species in Flora Iranica (Riedl 1968), with 39 species growing in Iran (Khatamsaz 2002, Attar and Jouharchi 2006, Attar 2007).

O. microcarpum Steven ex DC., of the sect. *Onosma*, subsect. *Haplotricha* (Boiss) Gürke., has wide distribution in Iran (Fig. 1) occurring in various environmental

	Population	Locality	Collector	Voucher No.
1	Kolakchal	Tehran, Kolakchal Mountain region	Mehrabian	HSBU-2010240
2	Neor	Ardebil, Neor lake, Mountain region	Mehrabian	HSBU-2010241
3	Avaj	Hamedan, Avaj to Razan	Mehrabian	HSBU-2010242
4	Tuchal	Tehran, Tuchal Mountain region	Mehrabian	HSBU-2010243
5	Belgheis	Zanjan, 25 Km Takab, Belgheis Mountain region	Mehrabian	HSBU-2010244
6	Taleghan	Ghazvin, Taleghan, Near the Talaeghan Dam	Mehrabian	HSBU-2010245
7	Gahvareh	Kermanshah, West Eslam Abad, Gahvareh	Mehrabian	HSBU-2010246
8	Tafresh	Markazi, Tafresh	Sheikakbari &Ghorbani	HSBU-2010247
9	Nikpey	Zanjan, Nikpey to Mahneshan, Mountain region	Mehrabian	HSBU-2010248
10	Paveh	Kermanshah, Paveh, Mountain region	Mehrabian	HSBU-2010259
11	Sohanak	Teharn, Sohanak	Mehrabian	HSBU-2010250
12	Ganjnameh	Hamedan, Ganjnameh	Mehrabian	HSBU-2010251
13	Khalkhal	Ardebil, Khalkhal to Givi	Mehrabian	HSBU-2010252
14	Arak	Markazi, Arak, Bone Village	Mehrabian	HSBU-2010253
15	Kohandan	Qom, Kohandan Mountain Region	Mehrabian	HSBU-2010254
16	Marivan	Kurdistan, Marivan	Mehrabian	HSBU-2010255
17	Dizeen	Tehran, Shemshak to Dizeen, Mountain Region	Mehrabian	HSBU-2010256

Table 1. Onosma microcarpa populations, their locality and vouchr No.

conditions. During our collection we encountered morphological variations among different geographical populations of this species, therefore attempted to study inter-populations morphological and molecular variations in this species.

A native species varies genetically in its adaptation to the particular localities and environmental conditions under which it grows. This results in a number of ecotypes of the same species or gradations (clines) between populations. Genetically based variations have been correlated with various environmental factors including light, water, temperature, rain, etc. (Rapson and Wilson 1992).

Molecular studies are very limited in the genus *Onosma*, mainly confined to AFLP (Amplified Fragment Length Polymorphism) study of genetic diversity in populations of *Onosma echioides* L. (Mengoni *et al*. 2006). In the present study, ISSR (Inter Simple Sequence Repeats) markers have been used to reveal populations genetic diversity. ISSR markers show high level of repeatability and have been used as useful

	1	2	3	4	5	6	7	8	9	10
Height (cm)	12-15	15.1-20	20.1-25	25.1-30	30.1-35	35.1-40				
No. of stem branches	1-3	4-6	6□							
Leaf basal length (mm)	25-35	35.1-45	45.1-55	55.1-65						
Basal leaf width (mm)	1-2	2.1-3	3.1-4	4.1-5						
Caule leaf length (mm)	16-26	26.1-36	36.1-46	46.1-56						
Caule leaf width (mm)	2.5-3.5	3.6-4.6	4.7-5.7	5.8-6.8	6.8□					
Calyx length (mm)	9-10	10.1-11	11.1-12	12.1-13	13.1-14	14.1-15	15.1-16			
Calyx width (mm)	2.6-3.6	3.7-4.7	4.8-5.8							
Teeth width (mm)	1-1.5	1.6-2	2.1-2.5							
Corolla length (mm)	12-13	13.1-14	14.1-15	15.1-16	16.1-17	17.1-18	18.1-19	19□		
Corolla width (mm)	1.8-1.9	2-2.9	3-3.9	4-4.9	4.9□					
Flower number	6-10	11-15	16-20	20□						
Inflorescent number	2-5	6-9	10□							
Bract length (mm)	9-11	11.1-13	13.1-15							
Bract shape	Lanceolate-Linear	Wide lanceolate								
Pedicle(mm)	1-1.5	1.6-2	2.1-2.5	2.6-3						
Anther long(mm)	4.9-5.9	6-6.9	7-7.9	8-8.9						
Style length (mm)	13-16	17-20	21-24	25□						
Seed length (mm)	2.5-3	3.1-3.6	3.6□							
Seed width (mm)	2.1-2.6	2.7-3.2								
Seed beak (mm)	1-1.1	1.2-1.3	1.4-1.5							

Table 2. Morphological characters used and their coding.

molecular markers in studying genetic diversity and species relationships (for example, Pharmawati et al. 2004, Dogan et al. 2007).

Materials and methods

Morphometry

Plant specimens collected from 17 *O. microcarpa* populations (Table 1), were use for morphological and molecular studies. Ten randomly selected plants were used for both morphological and molecular studies. In total 21 morphological characters (Table 2) were used for morphometry and coded accordingly.

For multivariate analyses the mean of quantitative characters were used, while qualitative characters were coded as binary/multistate characters. Standardized variables (mean = 0, variance = 1) were used for multivariate statistical analyses. The average taxonomic distance and Manhatan distance were used as dissimilarity coefficient in cluster analysis of morphological data (Podani, 2000). Principal components analysis (PCA) was performed to identify the most variable morphological characters among the populations studied and PCA plot of the components obtained were used to get species groupings (Podani 2000).

ISSR assay

Total genomic DNA was extracted from fresh leaves using the CTAB method by Murry & Tompson (1980) with the modification described by De la Rosa et al. (2002). Six ISSR primers used are $(GA)_9T$, UBC810, UBC811, UBC834, UBC849 and CA7GT commercialized by UBC (the University of British Columbia). PCR reactions were performed in a 25 μL volume containing 10 mM Tris–HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP; 0.2 μM of a single primer; 20 ng genomic DNA and 3 unit of Taq DNA polymerase (Bioron, Germany). Amplifications reactions were performed in Techne thermocycler (Germany) with following program: 5 min initial denaturation step 94° C, 30 s at 94° C; 1 min at 50° C, 1 min at 72° C. The reaction was completed by final extension step of 7 min at 72° C. Amplification products were visualized by running on 2% agarose gel, following ethidium bromide staining. Fragment size was estimated by using a 100 base pairs (bp) molecular size ladder (Fermentas, Germany).

ISSR bands obtained were treated as binary characters and coded accordingly (presence =1, absence = 0). Jaccard and Nei & Li similarity coefficients were used for ISSR data clustering (Podani 2000). UPGMA (Unweighted Paired Group with Arithmetic Average) and NJ (Neighbor Joining) clustering methods were performed for grouping of the populations by using NTSYS ver. 2 (1998) and PAUP vers. 4b (2000). In order to determine molecular difference between populations which were grouped in different clusters, AMOVA test was performed. (Podani 2000), by GE-NALEX 6 (Peakall and Smouse 2006).

Results

Morphometry

The *O. microcarpa* populations studied were distributed in 9 different provinces growing from north to side across 1200 km and from west to east across 860 km area (Fig. 1). These plants occurred in mountain slopes and road sides, growing in different climates (Table 3).

Population	Altitude	Latitude	Heigt	Habitat	climate
Kolakchal (Tehran)	35° 50′ 43 N′	51° 27′ 11′ E	2396m	mountain slope	semi arid - cold
Neor (Ardebil)	38° 00′ 49 N′	48° 24′ 43′ E	1950m	mountain slope	warm mediteranean
Avaj (Hamedan)	38° 04′ 46 N′	48° 21′ 08′ E	2360m	mountain slope	semi arid - ultra cold
Tuchal (Tehran)	35° 49′ 43 N′	51° 23′ 14′ E	2300m	mountain slope	semi arid- cold
Belgheis (Zanjan)	36° 43′ 29 N′	47° 27′ 27′ E	2600m	mountain slope	warm mediteranean
Taleghan (Ghazvin)	36° 08′ 20 N′	50° 31′ 24′ E	1850m	mountain slope	semi arid - cold
Gahvareh (Kermanshah)	34° 13′ 23 N′	46° 17′ 05′ E	1566m	mountain slope	warm mediteranean
Tafresh (Markazi)	37° 41′ 05 N′	50° 01′ 36′ E	2100m	mountain slope	semia arid - cold
Nikpey (Zanjan)	36° 43′ 46 N′	47° 53′ 38′ E	2200m	road side	semia arid - cold
Paveh (Kermanshah)	34° 54′ 42 N′	46° 26′ 39′ E	1474m	road side	semi mediteranean
Sohanak (Tehran)	35° 49′ 43 N′	51° 31′ 50′ E	2200m	mountain slope	semi arid - cold
Ganjnameh (Hamedan)	34° 44′ 46 N′	48° 29′ 57′ E	2136m	rocks of road side	semi arid - cold
Khalkhal (Ardebil)	37° 40′ 55 N′	48° 25′ 30′ E	1651m	road side	semi humid -cold
Arak (Markazi)	34° 02′ 37 N′	49° 39′ 58′ E	2180m	mountain slope	semi arid- cold
Kohandan (Qom)	34° 40′ 16 N′	50° 05′ 00′ E	2500m	mountain slope	semi arid- cold
Marivan (Kurdistan)	35° 24′ 59 N′	46° 22′ 31′ E	1950m	Road side	humid - cold
Dizin (Tehran)	35° 58′ 05 N′	51° 29′ 20′ E	2316m	Road side	semi arid- cold

Table 3. Geographical features of the populations studied.

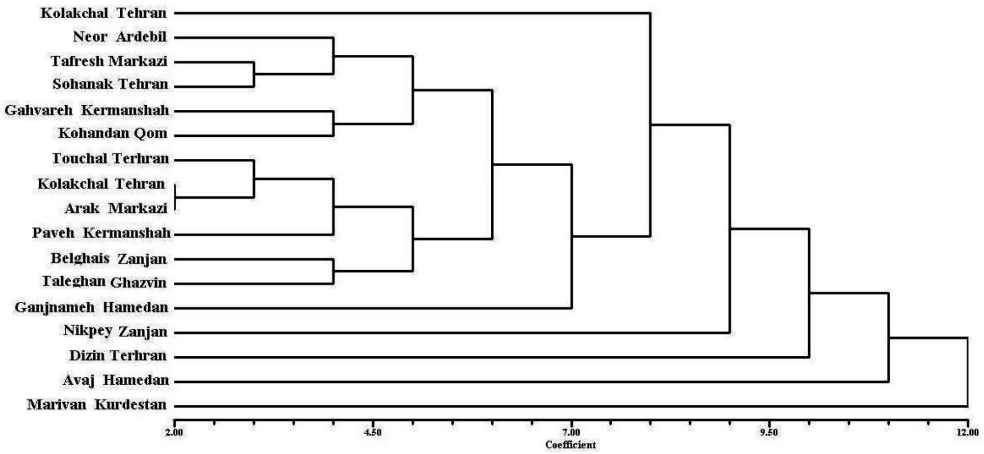

Fig. 2. NJ tree of morphological characters.

UPGMA and NJ clustering of morphological data produced similar results with NJ tree having a higher cophenetic correlation (r=0.98) and therefore is discussed here (Fig. 2). The populations of Marivan (Kurdestan), Avaj (Hamedan), Dizin (Tehran) and Nikpey (Zanjan) show a higher degree of morphological differences and stands apart from the other populations.

The populations of Belghias (Zanjan) and Taleghan (Ghazvin), Touchal (Tehran), Kolakchal (Tehran), Arak (Markazi), Paveh (Kermanshah), show similarity in morpho-

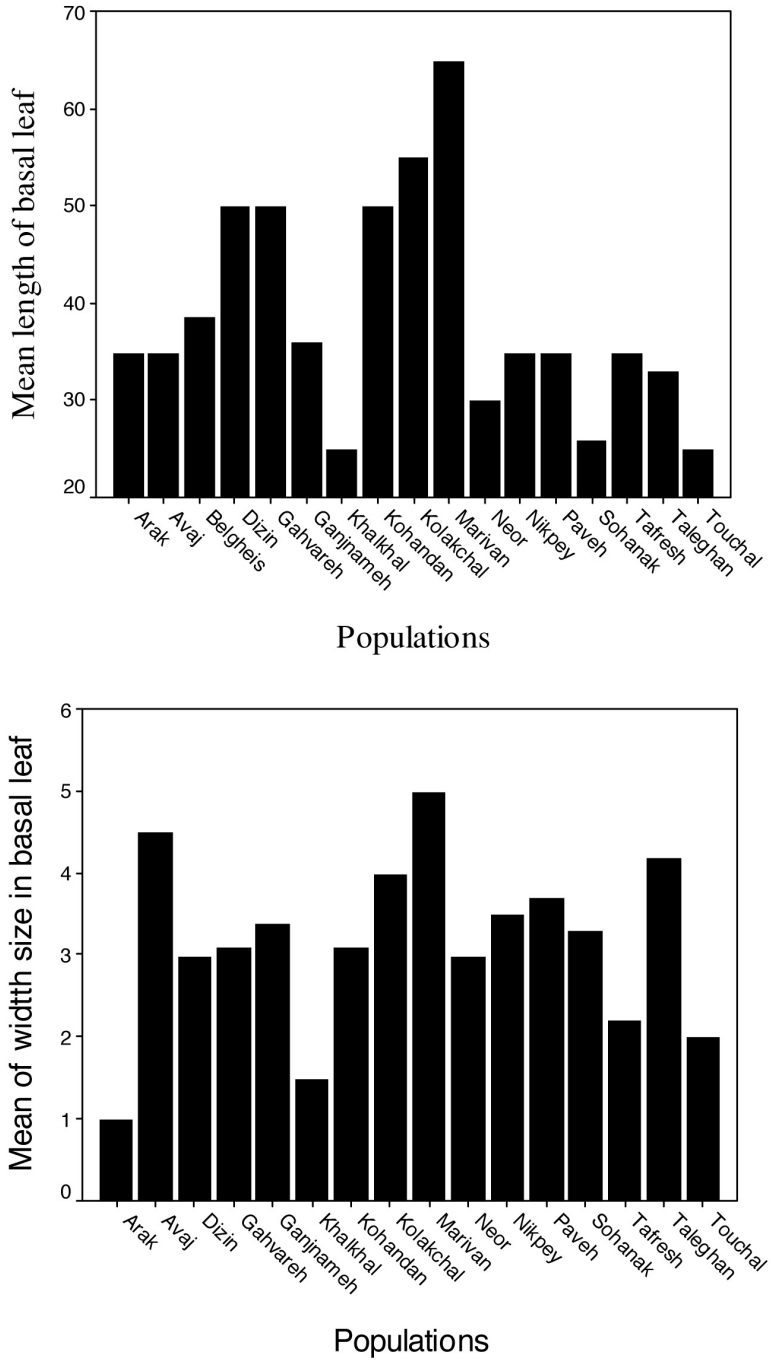

Fig. 3. Representative graphs showing the basal leaf length and width in populations studied.

logical characters and are placed in a single major cluster, in which Kolakchal (Tehran) and Arak (Markazi) populations show high affinity. These populations do not form a cline in their geographical distribution.

The populations of Neor (Ardebil), Tafresh (Markazi), Sohanak (Tehran), Gahvareh (Kermanshah) and Kohanak (Qom), form the other cluster based on morphological affinity. These two clusters are joined together with some distance, to which the population of Kolakchal (Tehran) joins with a greater distance (Fig. 2).

PCA analysis performed to identify the most variable morphological characters among *O. microcarpa* populations revealed that the first four PCA factors comprise about 60% of total variance (data not given). In the first factor with about 26% of total variation, characters like caule leaf size and width, calyx length and width as well as length and width of basal leaf has the highest positive correlation (>0.70) with this component, while in the second component with about 14% of total variation, corolla length and bract shape showed the highest negative correlation with the component (-0.60) and the No. of inflorescence showed the highest positive correlation (0.60). Therefore, these are the most variable morphological characters among the populations studied. Two populations of Marivan and Avaj showed significantly higher values for most of the morphological characters studied compared to the other populations (Fig. 3).

ISSR analysis

Out of 10 ISSR primers used (alone and in combination), 6 primers produced 67 polymorphic reproducible bands. Some common bands were observed in all populations studied, for example band 500 bp of the ISSR primer UBC-811, bands 270, 500 and 850 bp of the primer GA9C-GA9A. Some of the populations showed the presence of specific ISSR band/locus, for example, Arak population was the only population having ISSR band 350 bp of the combined primer UBC807-834, while Kolakchal population had specific band of 570 bp in this primer. Similarly, Avaj population was the only population showing the occurrence of band with 950 bp of the same combined primer.

Some ISSR bands were present in all populations except one, for example, ISSR band 750 bp of the of the combined primer UBC807-834 and band 400 bp of the primer UBC-811 were present in all populations except in Kolakchal population.

UPGMA and NJ clustering of ISSR data obtained produced similar results with NJ tree showing higher cophenetic correlation (r=0.98) and discussed bellow (Fig. 4). The populations of Kohandan (Qom) and Khalkhal (Ardebil) showed similarity in molecular features and are joined each other forming a separate cluster far from the other populations studied.

Kolakchal (Tehran) and Nikpey (Zanjan) populations join each other and form another distinct cluster. Four populations of Gahvareh (Kermanshah), Sohanak (Tehran), Taleghan (Ghazvin) and Belghais (Zanjan) show similarity and form the third major cluster. Two populations of Marivan and Avaj show affinity based on molecular analysis and are placed close to each other just similar to morphological data. Two other populations of Paveh (Kermanshah) and Arak (Markazi) also joing Marivan and Avaj with some distance, all together forming the fourth cluster. The populations of Tafresh (Markazi), Ganjnameh (Hamedan), Dizin (Tehran), Touchal (Tehran) and Neor (Ardebil) comprise the fifth cluster based o ISSR data.

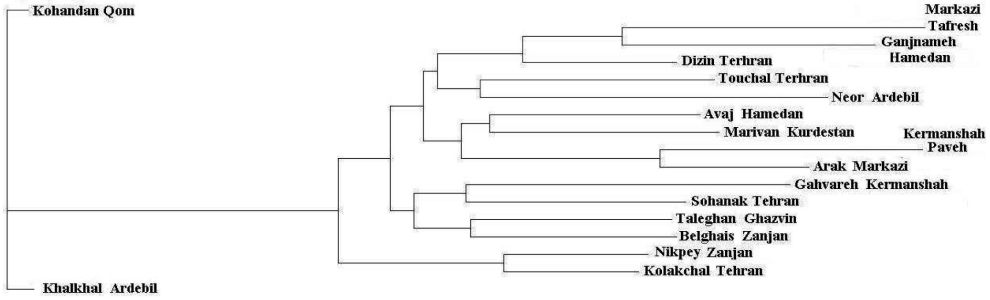

Fig. 4. NJ tree of ISSR data.

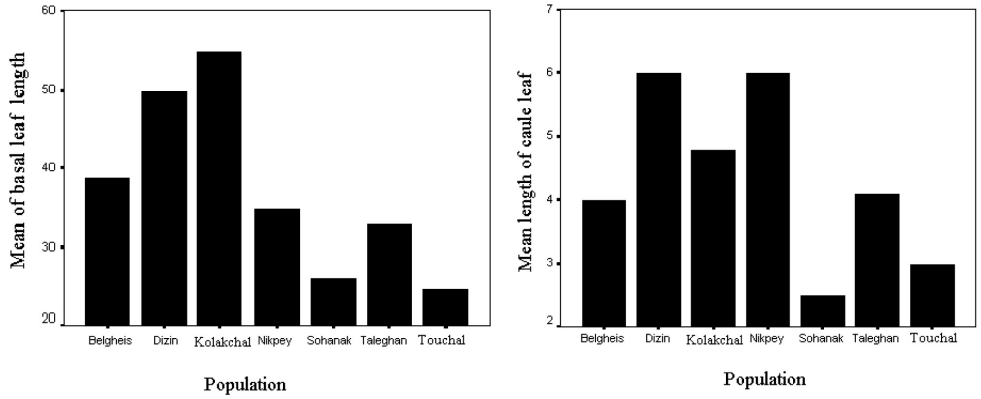

Fig. 5. Bar graph of basal and caul leaf length among the populations placed in geographical cline.

AMOVA test performed among the five clusters obtained showed a significant molecular difference among them. However, when similar test performed among populations of different provinces studied showed no significant difference and had almost similar values of Nei's genetic diversity (0.13-0.15) and Shannon index (0.14-2.00).

AMOVA test performed among the five clusters obtained showed 13% among cluster variation and 83% within cluster variation. Within clusters molecular variation ranged from 15.50 in cluster No. 2 (with two populations of Kohandan and Khalkhal) to 58.20 in cluster No. 5 (with five populations of Tafresh, Ganjnameh, Dizin, Touchal and Neor).

Discussion

Geographical distribution of the *O. microcarpa* populations studied comprises 8 different provinces in Iran having different climates indicating potential genetic

adaptation of this species to different environments. This is supported by molecular and morphological differences observed among these populations.

Geographical distribution of the populations studied form a continous cline in some parts of the country, for example populations of Belgheis, Nikpey (both from Zanjan province), Taleghan (Ghazvin), Dizin, Touchal and Kolakchal (all from Tehran province) are placed in a continues geographical cline. In such geographical clines we may expect gradual change in the morphological characters (clinal variation), but when we plot the graph of morphological chacters among these populations we do not observe a gradual change in the size from Tehran populations towards Zanjan as it is expected (For example, see Fig. 5).

A cline is variation in morphological, physiological and genetic features of organisms with ecological adaptability and is considered to be strong evidence of adaptation to geographically varying selection (Montesinos-Navarro *et al*. 211). Altitudinal gradients have been commonly used to study. These altitudinal gradients offer steep environmental gradients across short spatial distances and when accompanied with other environmental gradients bring up changes in the organisms features leading to the formation of new ecotypes in a species (Montesinos-Navarro *et al*. 211).

The occurrence of common ISSR bands in the populations studied indicate their affinity and to be the member of a single species, while the occurrence of specific bands in some of the populations indicated genetic diversity among populations.

Tree obtained from both morphological and ISSR data do not show any association between geographical area and the characters studied as the members of each cluster are from different geographical regions. For example, the five populations placed in the cluster No. 5 based on ISSR data i.e. Tafresh (Markazi), Ganjnameh (Hamedan), Dizin (Tehran), Touchal (Tehran) and Neor (Ardebil) differ in their localities, altitude and latitude, habitat and climate characteristics (Table 3). Tafresh population is from Markazi province and its plants grow on mountain slopes having semi-arid to cold weather, while Ganjnameh population is from the Hamedan province with its plants growing on road sides in semi-arid to cold climate. The same hold true for Neor population from Ardebil province with plants growing on mountain slopes with warm Mediterranean climate. The populations placed in other clusters also are from different provinces and their plants grow on different habitats and climates. However, Mengoni *et al*. (2006), studied genetic diversity in 8 populations of *Onosma echioides* in the north and central Italy and based on AFLP (Amplified Fragments Length Polymorphism) markers showed correlation between genetic and geographical distance. Difference in these two studies may be due to difference in genetic adaptation of the two Onosma species studied.

PCA analysis of morphological characters showed that the caule leaf size and width, calyx length and width as well as length and width of basal leaf, the corolla length and bract shape as well as the No. of inflorescence are the most variable morphological characters among the populations studied. These morphological characters are of taxonomic application in the genus *Onosma*, therefore if we encounter any population which differ in these morphological characters and also such difference is supported by genetic evidence, we may suggest a new ecotype or variety. As both morphological and ISSR trees show two populations of Marivan and Avaj are grouped together forming a distinct cluster. These populations also show higher values for most of the

morphological characters studied compared to the other populations. Therefore we may consider them to be a new form (either ecotype or variety).

AMOVA test performed among the five clusters obtained showed 13% among cluster variation and 83% within cluster variation. Within clusters molecular variation ranged from 15.50 in cluster No. 2 (with two populations of Kohandan and Khalkhal) to 58.20 in cluster No. 5 (with five populations of Tafresh, Ganjnameh, Dizin, Touchal and Neor). Mengoni et al. (2006), while studying genetic diversity in 8 populations of *Onosma echioides* in the north and central Italy by using AFLP markers reported genetic differentiation between populations and also noticed a high level of within-population genetic variance.

The result of present study therefore reveals inter-populational genetic and morphological differences in *O. microcarpa* but does not show any association between geographical distance and genetic/morphological variations. However, we possibly have different ecotypes, which should be studied in more detailed in future.

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Chromosome Markers

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Summary

A short review article on chromosome markers in plants. The contribution details a brief history of some significant markers and then expands on more recent work based largely on the use of *In Situ* Hybridization (GISH) and its application for revealing the genomic structure of allopolyploids and alien introgressions. The use of GISH in the *Lolium*/*Festuca* hybrids for introgression mapping, and for the identification of the stay-green gene, is considered in more detail.

Chromosome markers

Chromosome markers have a long history and utility in studies on genome organization, species relationships and gene mapping. A notable early example is that of Heitz in 1928 who used heterochromatic blocks in the chromosomes of *Pellia epiphylla* to track the integrity of chromosome structure throughout the cell cycle, and to determine that the chromosomes maintained their organisation and did not break up at interphase and then reassemble again at the next mitosis. Navashin (1934) later showed nucleolar dominance in Crepis hybrids, including *Crepis capillaris* x *C. parviflora*, where *Crepis capillaris* 'lost' its secondary constriction in the hybrid, and its nucleolus was absent at prophase of mitosis. But when the F1 was backcrossed to one of the parents the nucleolus reappeared, thus showing that it was suppressed rather than deleted: what we now call epigenetics. In the 1940s the pachytene chromosomes of *Zea maize* served as markers for chromosome mutations, based on identifying each chromomere, and the chromomere patterns, in all of the chromosomes (author, personal knowledge). McClintock used markers in the short arm of chromosome 9 to discover transposable elements (McClintock, 1951).

A major advance took place in the 1990s, with the advent of Genomic *In Situ* Hybridisation (GISH), which enabled the identification of the genomes comprising allopolyploid species. One of the earliest was the hybrid between *Lolium multiflorum* (2x) x *Festuca pratensis* (4x), clearly identifying the 7 green-coloured c-metaphase chromosomes of *L. multiflorum* and 14 yellow ones of *F. pratensis* (Thomas et al.

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1994). Later studies resolved the genomic constitution of hexaploid *Festuca arundinacea* (Humphreys MW, 1995), and many others. GISH, and ISH, also made it possible to discriminate alien chromosomes and their disposition at interphase, as well as chromosome segments, in addition lines, such as a single chromosome of rye in the background of hexaploid wheat (Mikhailova 1998). Such markers are based on the variation in the dispersed repetitive DNA in different, and even related species. The *Lolium*/*Festuca* species complex is the best known system for discriminating genomes in hybrids, as well as for GISH-based introgression mapping and for identifying introgressed genes (Jones et al. 2009). The basis of the scheme for introgression mapping in *Lolium*/*Festuca* is shown in Fig. 1.

Monosomic substitutions for all 7 *Fp* chromosomes have now been isolated, and identified by *Fp*-specific markers (Harper et al. 2011). The reciprocal of introgression of segments of *Lp* into *Fp* has also been achieved. Recombination at meiosis, as in the triploid hybrid in Fig. 1, along the entire length of *Lp*/*Fp* bivalents, also generates *Fp* segments, of varying sizes, as well as whole chromosomes in the BC1 backcross progeny. Promiscuous recombination thus enables the transfer of **any** *Fp* segment or gene into *Lp*, and despite the high level of recombination GISH readily discriminates the *Lp* and *Fp* genomes. A recombination series of *Fp* segments of differing sizes can thus be assembled, and due to the high level of polymorphism (RFLP, AFLP, SSR) between *Fp* and *Lp* mapping of the introgressed segments can be undertaken. By comparing the physical size of introgressed *Fp* segments with the presence or absence of *Fp*-specific polymorphisms the physical location of genetic markers can be determined.

The utility of introgression breeding, and the mapping of introgressed segments, has been exploited to map and clone the stay-green gene of *Festuca pratensis*. By repeated backcrossing the size of the *Fp* introgressed segment carrying the green gene (*y*), in the *Lp* background, was located to the terminal region of a single pair of *Lp* chromosomes. *Fp*-specific AFLP markers enabled an initial map of the introgressed segment carrying the ***y*** gene. Comparative mapping then revealed that the ***y*** locus is associated with *Lp* chromosome 5, which is syntenic with a region of rice chromosome 9. Further fine mapping of *Lp*/*Fp* using rice markers narrowed the candidate gene down to small number on a single rice BAC, with the most likely candidate sequence being Os09g3620, which is homologous with Arabidopsis At4g22920. An RNAi knockout of the Arabidopsis homologue confirmed the identity the of the stay-green gene (Jones et al. 2009).

Another significant outcome of GISH markers was the finding that in tetraploid hybrids of *L. perenne* (2n=4x=28) x *F. pratensis* (2n=4x=28) the balance of the chromatin, in advanced generations of open pollination, was not equal. The genome balance was in favour of *Lp*, in the ratio of about 18:10 of *Lolium* to *Festuca* chromatin (Canter et al. 1999). This mysterious situation was later confirmed by Zwierzykowski et al. (2006) who followed the this genomic drift through six open pollinated generations of a *Festuca pratensis* x *Lolium perenne* tetraploid hybrid. In the F2 generation the balance of chromatin, as determined by GISH, was equal for the two genomes, but by the F6 the balance was 60:40 in favour of *Lolium* (Fig. 2). It seems, from various hybrids, that *Lolium* is always the dominant genome and *Festuca* the one that is progressively replaced. The mechanism of this genome drift remains to be determined.

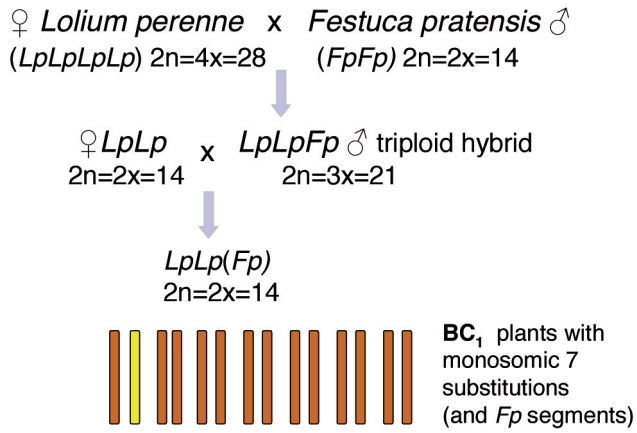

Fig. 1 Introgression mapping in Lolium/Festuca, based on King et. al 1998. Heredity 81: 462-467.

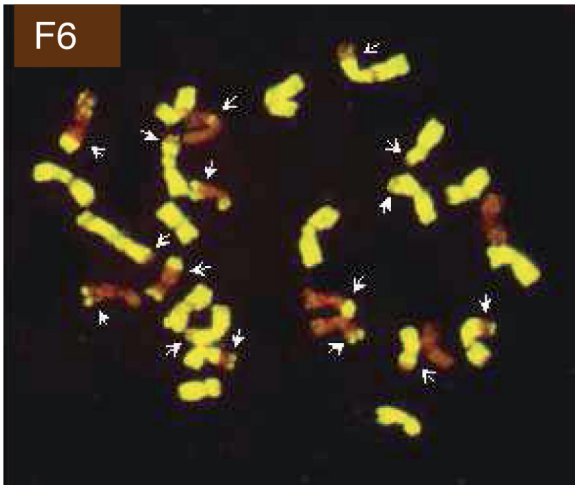

Fig 2. The F6 of the tetraploid hybrid between F. pratensis x Lolium perenne (2n=4x=28), showing the dominance of the Lp (yellow probe) over the Fp chromatin in the proportion 60:40. From Zwierzykowski et al. 2006.

Chromosome-specific BACs have found utility in small genome species for chromosome painting, and for analyzing the arrangement of chromosomes in interphase nuclei.

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Mapping of the sex trait and sequence analysis of two linked genomic regions in Populus tremuloides

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Summary

The mechanism of sex determination in *Populus* is still an open question. In the sequenced female *P. trichocarpa*, sex determination was located to the telomeric region of chromosome XIX. We mapped sex-linked SSR and SNP markers to a central position on the respective linkage group in a male *P. tremuloides* clone and localised a recombination-suppressed sex-linked region, spanning about 1.67 million bp in the corresponding central region of *P. trichocarpa* chromosome XIX. Two BAC clones representing parts of this sex-linked region were isolated and sequenced. The BAC sequences include genes showing high similarity to genes located in the centromeric/pericentromeric region of *P. trichocarpa* chromosome XIX. Moreover, a high portion of repetitive sequences, mainly represented by *Ty3/Gypsy* LTR retroelements, was detected in the BAC sequences. No likely candidate genes for sex determination were found within the sequenced *P. tremuloides* BACs, but several potential candidates were identified within the central region of *P. trichocarpa* chromosome XIX.

Introduction

Species of the genus *Populus* are dioecious, but the mechanism of sex determination - though supposed to be genetically controlled - has not been precisely identified (Tuskan *et al.* 2012). Yin *et al.* (2008) mapped sex as a genetic marker on a terminal position of chromosome XIX in the *P. trichocarpa/P. deltoides*-based *Populus* consensus map (sex was mapped in a *P. deltoides* x (*P. deltoides* x *P. nigra*) cross). Based on these results and on *P. trichocarpa* sequence data, the authors suggest that *P. trichocarpa* chromosome XIX seems to be an incipient sex chromosome

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with female heterogamety. Sex as a genetic marker has also been mapped to several genetic linkage maps of other species of the genus *Populus*. Thereby all sex-linked genetic markers have consistently mapped to chromosome XIX, supporting a central role of this chromosome in sex determination. However, among the different *Populus* species, the respective sex-determining loci seem to be located on different regions of chromosome XIX and seem to indicate different heterogametic sexes (Gaudet 2006; Gaudet *et al.* 2008; Pakull *et al.* 2009 and 2011; Paolucci *et al.* 2010).

In *P. tremuloides,* three SSR markers (derived from the *P. trichocarpa* genomic sequence of chromosome XIX; Tuskan *et al.* 2006), which completely co-segregate with sex, were identified (Pakull *et al.* 2011). Blast analysis against the *P. trichocarpa* genome (v2 available at http://www.phytozome.org/) localised the markers in a central region on chromosome XIX, spanning about 670,000 bp (positions 8,074,724 bp to 8,746,182 bp; Pakull *et al.* 2011).

In this study, we aimed to extend the fully sex-linked region in *P. tremuloides* by mapping of SNP markers. Two *P. tremuloides* BAC clones were analysed representing parts of this sex-linked region. The BAC sequences and the *P. trichocarpa* region corresponding to the sex-linked region in *P. tremuloides* were screened for candidate genes for sex determination.

Materials and Methods

Genetic mapping of the markers was carried out in a *P. tremula* L. (Brauna11) x *P. tremuloides* Michx. (Turesson141) cross (Pakull *et al.* 2011). The F1 mapping population consisted of 45 female and 74 male individuals. SNP markers were developed based on *P. trichocarpa* gene sequences by PCR and Sanger sequencing. Sequences were checked for SNPs that were heterozygous in Turesson141 and homozygous in Brauna11, using Seqman Pro 7.1.0 (DNAStar, Madison, USA). These SNPs were analysed in the mapping population using either PCR with SNP-specific primers or restriction of PCR products with SNP-specific endonucleases (Table 1). Mapping of the SNP markers on the paternal linkage group XIX of a *P. tremula*/*P. tremuloides* map was carried out as described in Pakull *et al.* (2011). Joinmap 4.1 (Kyazma B.V., Wageningen, The Netherlands) was used for map calculations.

The BAC library used (55,296 clones, 8X haploid genome equivalents) was constructed by Fladung *et al.* (2006) and was based on the *P. tremuloides* clone Turesson141, the male parent of the mapping population. Construction of BAC-DNA filters is described in Fladung *et al.* (2006). Probe fragments were amplified by standard-PCR using genomic DNA as template (primer sequences for BPTGG82-probes: for: 5'-CTGACTGAATACAACCGAAATGCG-3', rev: 5'-CTTTGAGGAGATGATATGCAACC-3'; primer sequences for BPTTG60-probes: for: 5'-AGCTCATGCCTGCACTCACCG-3', rev: 5'-CTTTGCACGTTAATAAGGAGACTG-3'). DNA filters were screened with radio-labelled DNA probes using standard procedures. BAC DNA was isolated (PSI Ψ Clone BAC DNA-kit, Princeton Separations) and used for 454-sequencing. Tagged libraries (single end) were generated from the fragmented, size-fractionated DNA of each BAC and the libraries were sequenced on a GS FLX (Roche/454; Titanium run; GATC Biotech AG, Konstanz, Germany). The 454-reads were assembled by GATC Biotech AG (Konstanz, Germany) using Newbler (454 Life Sciences). An alternative assembly was done using Mira (Chevreux *et al.* 2004). Scaffolds were generated by

Marker Name	Marker type	Polymorphism (Tur141)	Analysis method	Position (Start forward-primer)	Gene	Number of recombinant individuals (of 119)
BDP12	SNP	TATGATATGA-(C/T)(A/G)-TTTGGCACTG	SNP-specific primer	7069259	POPTR_0019s06340	0
BPCA90	SSR	CA-SSR	PCR	8074612		0
BPTGG82	SSR	TGG-SSR	PCR	8550308	POPTR_0019s07520	0
BPTTG60	SSR	TTG-SSR	PCR	8746069	POPTR_0019s07660	0
BPTG50	SSR	TG-SSR	PCR	8901872		1
BPGA41	SSR	GA-SSR	PCR	9824723		7

SNP and SSR markers (Pakull *et al.* 2011) were derived from the *P. trichocarpa* sequence of chromosome XIX (genome assembly v2 at Phytozome v7).

Table 1 SNP and SSR markers analysed in the P. tremula x P. tremuloides cross

SeqManPro v8.1.5 (Lasergene software suite DNASTAR, Madison, USA) and then combined to complete BAC sequences by PCR-amplification and Sanger sequencing of the gaps. *Ab initio* gene prediction with FGENESH (Salamov and Solovyev 2000), GeneScan (Burge and Karlin 1997) and Augustus (Stanke *et al.* 2004) was performed using the related web tools. The predicted gene models were annotated by BlastP of the peptide sequences against NCBI nonredundant protein sequences.

Results

Extension of the sex-linked region in a P. tremula x P. tremuloides cross

The fully sex-linked region so far defined by SSR markers (Pakull et al., 2011) was further extended by SNP markers. We mapped fully sex-linked SSR (Pakull et al., 2011) and SNP markers to a central position on the respective linkage group in a male *P. tremuloides* clone (data not shown). Table 1 presents the results of a sex linkage analysis of selected markers. The SNP marker BDP12 and three SSR markers co-segregate with sex in the analysed *P. tremula* L. (Brauna11) x *P. tremuloides* Michx. (Turesson141) cross. BPTG50, showing a single recombinant individual defines the lower border of the sex-linked region. The identified fully sex-linked *P. tremuloides* markers span 1.67 million bp when mapped to the *P. trichocarpa* genomic sequence (Table 1). So far, the most promising candidates for sex determination within the correspondent *P. trichocarpa* genomic region are POPTR_0019s07500.1 which is weakly similar to AT5G20930 (annotated as TOUSLED and involved in flower development) and POPTR_0019s07600.1, highly similar to AT1G65060.1 which is annotated as 4-coumarate-CoA ligase in *A. thaliana* (potentially involved in pollen exine formation).

Isolation, sequencing and annotation of two P. tremuloides BAC clones

Radio-labelled DNA probes (about 0,6-1 kb in size) including the sex-linked SSR markers BPTGG82 and BPTTG60 (Pakull *et al.* 2011) were employed to isolate parts

Gene	TSS	PolyA	Direc-tion	Ex	Predictor	Annotation (Phytozome/NCBI*)	e-Value
G1	6314	471	rev	12	FGENESH (*Medicago*)	TLC ATP/ADP transporter (POPTR_ 0019s07520.1)	0
G2	10756	14484	for	3	Augustus (*Arabidopsis*)	Possible lysine decarboxylase with PF03641 (POPTR_0001s27250.1)	$3E^{-42}$
G3	17113	23624	for	4	FGENESH (*Medicago*)	Hypothetical Pol protein VITISV_020846, *V. vinifera* (CAN65719.1)*	0
G4	23862	25131	for	2	FGENESH (*Medicago*)	Possible lysine decarboxylase (POPTR_0006s22120.1)	$3E^{-29}$
G5	30199	33145	rev	3	FGENESH (*Medicago*)	Integrase, *P. trichocarpa* (ABG37658.1)*	$9E^{-116}$
G6	35188	38782	for	3	FGENESH (*Medicago*)	Predicted polyprotein (Panther:10178) with retrotransposon gag protein (PF03732) and zinc knuckle (PF00098) protein domains (POPTR_1126s00200.1)	E^{-105}
G7	39238	44086	for	3	FGENESH (*Medicago*)	Hypothetical protein, *V. vinifera* with Retrotransposon gag protein domain (Pfam03732) among others (XP_002268669)*	0

Genes are presented as predicted transcripts from transcription start site (TSS) to polyadenylation site (PolyA). Positions are related to the BAC nucleotide sequence (see also Fig. 1). Ex: number of exons. Genes were annotated by BlastP of the related peptide sequence against *P. trichocarpa* protein sequences (JGI v2.2 gene annotation of assembly v2 at Phytozome v7) or NCBI* non-redundant protein sequences, if Phytozome provided no significant hit of e-values smaller than E^{-20}.

Table 2 Predicted genes in BAC96P5a

of the sex-linked region from a *P. tremuloides* BAC library (Fladung *et al.* 2006). BAC110J22, potentially defining the "Y"-haplotype, and BAC96P5a, potentially representing the "X"-haplotype were selected from the BAC library for sequence analysis. Complete BAC sequences of 47.362 kb for BAC96P5a (GenBank accession AC246528.1) and 53.796 kb for BAC110J22 (GenBank accession AC246527.1) were created by 454-sequencing (454-reads available at EBI SRA-project; http://www.ebi.ac.uk/ena/data/view/ERP001155). Contig sequences are accessible via GabiPD (Riano-Pachon *et al.* 2009; http://www.gabipd.org). All Sanger sequences related to the BAC clones are available at GenBank (dbGSS_Ids 32995072 to 32995093).

We attempted an *ab initio* draft annotation of both BAC sequences. Predicted gene models (Tables 2-3) were mapped onto the BAC nucleotide sequences (Figs. 1-2). Considering only non-transposable element sequences, three genes were identified which are very similar to genes annotated in the central region of *P. trichocarpa* chromosome XIX: a TLC ATP/ADP transporter gene similar to POPTR_0019s07520 (BAC96P5a_G1); a gene encoding a GAT and a VHS domain (BAC110J22_G1) which is similar to POPTR_0019s07660 and POPTR_0019s07670, and a potential peptidyl-tRNA hydrolase (BAC110J22_G3) similar to POPTR_0019s07680 (Figs. 1-2, Tables 2-3). All other predicted genes are probably of retrotransposal origin.

Gene	TSS	PolyA	Direc-tion	Ex	Predictor	Annotation (Phytozome/NCBI*)	e-Value
G1	14754	6087	rev	3	FGENESH (*Vitis*)	Putative protein with GAT domain (PF03127) and VHS domain (PF00790) (POPTR_0013s07880.1)	0
G2	25178	23612	rev	3	FGENESH (*Vitis*)	No hit	
G3	27631	33224	for	8	FGENESH (*Vitis*)	Similar to expressed protein in *A. thaliana* (co-ortholog (1of 2) of At5g16870, At3g03010) with Peptidyl-tRNA hydrolase PTH2 domain (PF01981) (POPTR_0019s07680.1)	$3E^{-89}$
G4	37174	39941	for	2	FGENESH (*Vitis*)	Integrase, *P. trichocarpa* (ABG37658.1)*	E^{-146}
G5	40010	41307	for	1	FGENESH (*Vitis*)	Possible lysine decarboxylase (PF03641) (POPTR_0006s22120.1)	$7E^{-57}$
G6	44589	52193	for	4	GeneScan (*Arabidopsis*)	Gag/pol polyprotein, 3'-partial, putative, *S. demissum* (ABI34371.1)*	$5E^{-38}$
G7	52722	53780	for	1	GeneScan (*Arabidopsis*)	Predicted protein, *P. trichocarpa* with Ribonuclease H domain (IPR002156) (XP_002338691.1)	E^{-91}

Genes are presented as predicted transcripts from transcription start site (TSS) to polyadenylation site (PolyA). Positions are related to the BAC nucleotide sequence (see also Fig. 2). For further legend see Table 2.

Table 3 Predicted genes in BAC110J22

Repetitive sequences in the sex-linked P. tremuloides region

RepeatMasker (http://www.repeatmasker.org/) reported a high portion of retrotransposable and repetitive elements in both BACs. The mean frequency of tandem repeats as identified by Tandem Repeats Finder (Benson 1999) was higher in the BAC sequences (one repeat per 1.9 kb) than in the chromosome XIX sequence (one repeat per 2.3 kb; data not shown).

Both internal parts and LTRs of autonomous retroelements of the *Ty3/Gypsy* family (Gypsy Database v2.0; Llorens *et al.* 2011) were identified (Figs. 1-2). Based on these results, retrotransposal elements cover approximately 87% of the BAC96P5a sequence and 41% of BAC110J22. More than half of the *Ty3/Gypsy*-retrotransposons included in the BACs shared sequence homologies to the *Athila* clade from *Arabidopsis* (Figs. 1-2).

The accumulation of tandemly repeated sequences and LTR retrotransposons, predominantly of the *Ty3/Gypsy* class (see above) are characteristics of centromeric/pericentromeric regions which feature a heterochromatic structure (Wu *et al.* 2004; Ma and Bennetzen 2006).

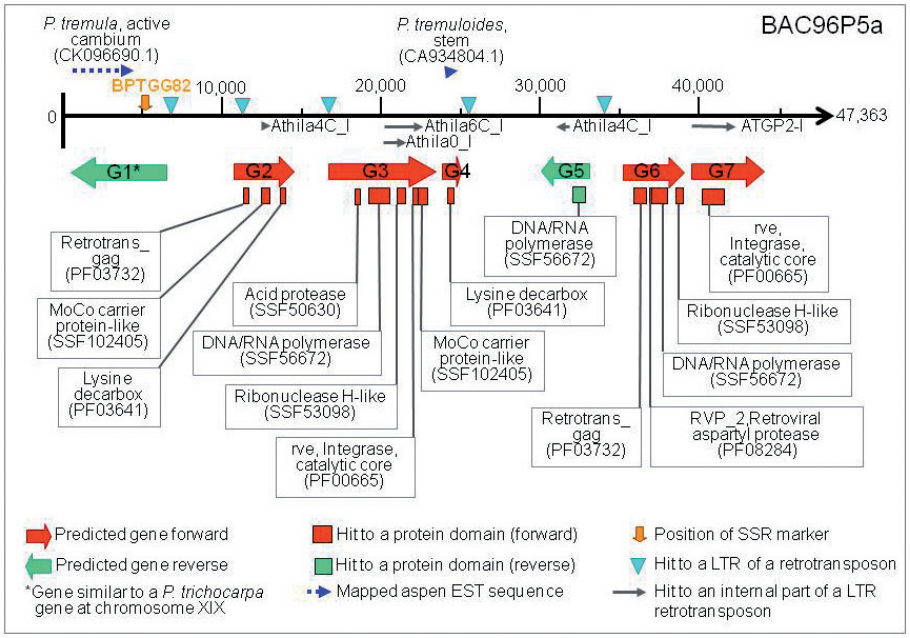

Fig. 1 Structural organisation of the sex-linked P. tremuloides BAC clone BAC96P5a. Nucleotide sequences of predicted transcripts (Table 2) were mapped from transcription start site to polyadenylation site. Hits to PFAM domains and protein superfamilies with e-values <E-10 as well as significant RepeatMasker hits to retrotransposal elements are shown. BlastN hits of aspen ESTs with at least 99% identity to the BAC sequence were mapped. Representative BlastN hits to LTRs (Gypsy database) with e-values smaller 2.5 were mapped.

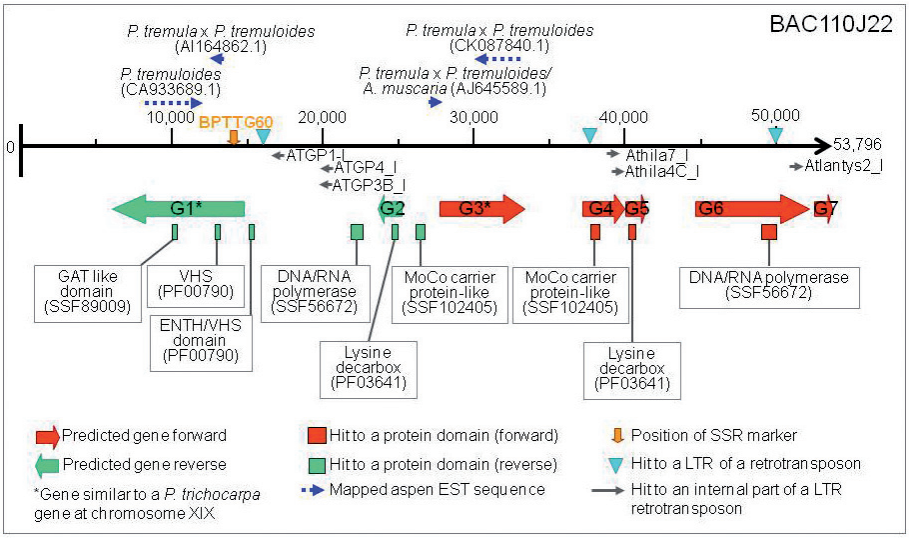

Fig. 2 Structural organisation of the sex-linked P. tremuloides BAC clone BAC110J22. Predicted transcripts according Table 3. See Figure 1 for further legend.

Conclusions

We suggest that the sex-determining region on *P. tremuloides* chromosome XIX is centromeric/pericentromeric and will therefore have suppressed recombination. Fully sex-linked *P. tremuloides* markers span about 1.67 million bp in the central region of *P. trichocarpa* chromosome XIX. A few candidate genes showing homology to genes known to be involved in floral determination or pollen development (e.g., TOUSLED, 4-coumarate-CoA ligase) were identified within the accordant *P. trichocarpa* genomic region. A comparative transcript expression analysis between male and female individuals during flower development is planned for these candidate genes. Ultimately, cloning selected candidate genes and experiments trying to verify the effect of transgenic over expression- and/or knockdown on the expression of sex will be required.

The gene models annotated in both *P. tremuloides* BAC sequences did not point to an obvious candidate gene for sex determination. This was not unexpected, because the BAC sequences represent only sub-regions of the complete sex-linked region. Thus, sequencing of the complete sex-linked region in *P. tremuloides* is warranted in the search for additional candidate genes. Once available, the centromeric chromosome XIX sequence of the *P. tremuloides* male Turesson141 clone can be compared to the telomeric sequence of the *P. trichocarpa* female Nisqually1 clone to check whether or not both regions contain orthologous genes.

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Mabc in rice breeding for submergence tolerance in vietnam

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Summary

Agriculture is affected by global climate change through various pathways. Sea level rise, storm surge and flooding, which result from climate change, adversely impact rice production in many regions in Vietnam. Contributed to solve that problem, we developed a new rice variety from KDDB into the one containing Sub1 QTL that can tolerate with submergence while maintaining its original characteristics preferred by farmers and consumers. The results proved that MABC aids in the transfer of target segments and may improve the recovery of the recipient genome KDDB if background selection is employed. The function of QTL Sub1 was affirmed via submergence tolerant stress in green house and on the field conditions.

I. Introduction

In Vietnam, rice is the number one agricultural product, not only for second largest exporter but also accounts for 90% of national food production. Of the 4.5 million ha rice area, more than one million ha is affected by flooding, remains for at least one-two weeks in many part of the country. Depending on the timing of flood with respect to growth stage, shorter duration and shallow flashfloods can result in less than 10% production losses while deeper and stagnant water with two weeks' duration and >100 cm depth can cause damage ranging from 40% to 77% (Manzanilla, D.O. et al., 2011)[2]. Development of submergence tolerant varieties is generally considered as the most effective entry point for improving productivity of rice varieties damaged from typhoon and flash flood, and it is also the cheapest option for farmers. This study aim at transfer Sub1 from IR64Sub1 variety, a major QTL on chromosome 9 explaining almost 70% of the phenotypic variance (Xu and Mackill 1996; Xu et al. 2006)[4,5], into the widely-grown rice cultivar KDDB in Vietnam.

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II. Materials and methods

II.1.Materials

Donor of SUB1 was the IR64 SUB1 cultivar – derived submergence tolerant breeding line was used. This variety was developed that can yield 4 ton/ha under water depth from 0.8 – 1 meter for 20 – 25 days in Vietnam conditions (Nguyen Thi Lang, 2010)[1].

The recipient variety KDDB (mutanced from KDDB)

II.2. Methods

*Marker genotype data: was obtained by running SSR markers using 15 µL PCR reactions on 96-well plates. After initial denaturation for 4 min at 94^0C each cycle comprised 1 min denaturation at 94^0C, 1 min annealing at 55^0C, and 1 min extension at 72^0C with a final extension for 5 min at 72^0C at the end of 35 cycles (Eppendoft thermal cyclers). The PCR products were analyzed by electrophoresis on agarose gel before loading into 4.5% and 6% acrylamide gel followed by silver stainning steps or syber safe and scoring.

*Data analysis: The molecular weights of the different alleles were measured using Alpha Ease Fc 5.0 software. The marker data was analyzed using the software Graphical Genotyper (GGT 2.0) (Van Berloo, 2008)[3]. The homozygous recipient allele, homozygous dominant allele and heterozygous allele were scored as 'A', 'B' and 'H'. The percent markers homozygous for recipient parent (%A) and the percent recipient alleles including heterozygous plants (%R) were calculated.

*Screening of precision introgression lines for submergence tolerance

Submergence screening was performed in the greenhouse at Agricultural Genetics Institute, Hanoi- Vietnam following standard protocols (Xu et al. 2000). Seeds of the BC2F2 generation with parents and susceptible check IR42 were germinated in rows in 35cm/20 cm trays with three replications. Fourteen-day-old seedlings were submerged for 14 days. The survival percentage and elongation ratio of plants were taken 21 days after de-submergence.

III. Results

BC1F1 generation of the backcross (KDDB x IR64Sub1)

Total of 500 BC1F1 individuals were used for screening the Sub1 region (primer ART5 and SC3). From the first result, 250 individuals were selected for recombinant and background selection.

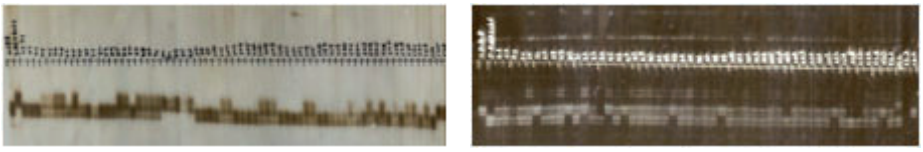

Figure 1: Screening BC1F1 individuals (KDDBxIR64Sub1) using primer SC3 (left) and primer RM105 (right)

Lane 1: KDDB, Lane 2: IR64Sub1, Others lanes: BC1F2 individuals

After that, there were 6 BC1F1 individuals selected for developing the BC2F1 generation.

BC2F1 generation of the backcross (KDDB x IR64Sub1)

Using the same procedures, we used 48 polymorphic primers for BC2F1 screening. Figure 2 was the result of BC1F2 genotyping. Finally, we can chose these plants for backrossing in the BC2F1 generation. They are plants number 259, 389, 395, 404, 450, 452. All these plants having the region of Sub1 and maximum background for the recurrent desired up to 89.36%. For these plants, we found out the recombinant at the position of Sub 1 QTL on the above plants.

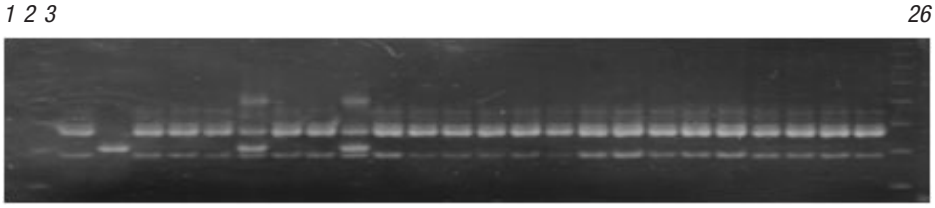

Figure 2: Screening 22 BC2F1 recombinant individuals (KDDB/IR64Sub1) using primer RM25. Lanes 1,26: 25bp marker, ,Lane 2:KDDB, Lane 3:IR64Sub, Lanes 4-25: BC2F1 individuals

For the development of KDDB-Sub1, RM24013 (9.4 Mb) was used as flanking marker at proximal end and RM105 (5 Mb) at distal end. Foreground selection was confirmed by the use of markers from the SUB1 genes ART5 (6.3 Mb) and SC3(6.6 Mb) in all the plants. The used of two precise primers located in the SUB1 region absolutely resulted in minimized the introgression size of the SUB1 in KDDB variety. It also reduced the crossing over between the gene region, which decreased the false-positive results in foreground selection. The SUB1 introgression gene will be used for further assessment in the BC4F1 and BC3F2 generations.

***** Submergence screening

The selected BC2F2 and BC3F1 lines showed submergence tolerance just after de-submergence and after recovery against submergence stress of 21 days. The submergence tolerance scores of these BC2F2 lines were similar to those of the tolerant donor IR64SUB1. The result confirmed the introgression ofSUB1 QTL in these lines. The situation of heterozygous SUB1 in BC3F1 resulted in the lower survival scores than the homozygous SUB1 in BC2F2 lines.

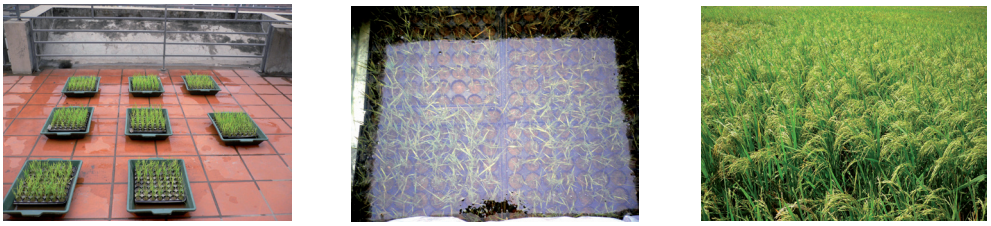

Figure 3: Screening for submergence tolerance in greenhouse and after that they were growing in the field – spring season 2012.

The agronomic traits of the potential lines BC2F3 and BC3F2 harvested in this crop season showing almost the same compare to KDDB original.

The further analysis will be performed in the next season.

IV. Conclusion

The present study was successfully adopted to introgress SUB1 into KDDB within a short time frame. This will become increasingly important as other desirable genes are introduced into KDDB. It is expected that the newly developed Sub1 lines will be able to increase rice production in the submergence areas of Vietnam in the very near future to cope with climate change in Vietnam.

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VI. Acknowledgements

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Marker Assisted Selection of the Solanum stoloniferum Based PVY Resistance in the Breeding Material of Keszthely

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Summary

Current objective of potato breeders is the development of varieties having resistance against major pathogens. One of the most important pathogens causing serious yield loss is PVY. During our investigations RAPD markers were developed for the Ry_{sto} gene, which is specific to the breeding material of Keszthely. Two of these markers were converted to SCAR marker. Using the marker ***ST1*** (1.3 cM to the target gene) 21 varieties/breeding lines, 174 individuals of 4 crossing families of Potato Research Centre of Keszthely were tested for the presence of the marker. Out of 21 genotypes 11 carried the marker while 10 not. Based on previous knowledge of their resistance to PVY we could calculate the selection efficiency of the marker. In case of segregating populations we have got 1:1 ratio of resistant:susceptible genotypes proving the simplex nature of Ry_{sto} gene in the resistant parents.

Introduction

Cultivated potato (Solanum tuberosum) is one of the world's most productive, nutritious, and tasty vegetables. To develop new cultivars having complex resistance against major biotic and abiotic stresses is in the stream line of current potato breeding. One of the economically most important potato pathogen is the potato virus Y (PVY, Fig. 1.). The highest level of resistance against PVY, extreme resistance is coded by Ry genes and can be found in several wild Solanum species (Ross 1986). New resistance genes are still being discovered (Barker, et al., 1996) and there are, no

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Figure 1.: Symptoms of PVY infection on potato tuber (left) and leaves (right)

doubt many others are still undiscovered. Introduction of wild species into cultivated potato allowed the obtaining of new resistance sources providing long-term effects against viruses, and further these are useful in breeding of new varieties (Heldák et al. 2009). The Ry_{sto} gene originating from *S. stoloniferum*, a wild potato species, confers extreme resistance to Potato virus Y (PVY) in potato. We developed a molecular marker, ST1, that shows 1.3 cM linkage to the Ry_{sto} gene in a mapping population of cv. White Lady x S440 (a breeding line). Here we report the usability of this marker for discrimination of PVY resistant and susceptible genotypes in a practical potato breeding programme.

Materials and methods

Plant material

In order to check the diagnostic value of the SCAR marker ST1, 12 potato varieties and 9 breeding lines with known phenotype to PVY infection were tested (Table 1.). Later four crosses 01.607 x (Lady Rosetta x S440); WL x W870; WL x 97.1011; WL x Latona) with total 174 F_1 progenies were also screened with the marker to identify resistant and sensitive genotypes (Table 2). The plants used for analysis were grown in a vector-free greenhouse at 20–23°C under natural illumination and were maintained in vitro, too.

Molecular examinations

The Ry_{sto} gene was tested by using the SCAR marker ST1. The *Phare Plant Direct PCR Kit* (Finnzymes) was used to amplify the marker. We collected 2 mm diameters leaves from each genotype and crashed in 10 µl Dilution Buffer. From this mixture 0,6 µl was used for each PCR reaction. The PCR mixture contained 0,6 µl DNA, 7 µl nuclease free water, 1-1 µl from the primer pairs (100pm/µl), 10 µl PCR puffer, and 0,4 µl Hot Start DNA Polymerase (Finnzymes). The fragments were visualized on agarose gel of 1,5% (Promega) stained with ethidium-bromide.

Cultivar/Breeding line	Resistance gene	Presence/absence of ST1 marker
Whie Lady	Ry_{sto}	+
Latona	Susceptible	-
Bettina	Ry_{sto}	+
Kánkán	Ry_{sto}	+
Ciklámen	Ry_{sto}	+
Rioja	Ry_{sto}	+
Somogyi kifli	Susceptible	-
Chipke	Ry_{sto}	-
Vénusz Gold	Ry_{sto}	+
Katica	Ry_{sto}	+
Hópehely	Ry_{sto}	+
Góliát	Ry_{sto}	-
S438	Susceptible	-
01.739	Susceptible	-
97.1011	Susceptible	-
S440	Susceptible	-
83.67	Ry_{sto}	+
87.3143	Ry_{sto}	+
06.325	Susceptible	-
01.607	Ry_{sto}	+
W870	Susceptible	-

Table 1. Summary of MAS in potato cultivars and breeding lines.
'-' absence of marker ST1,
'+' presence of marker ST1, recombinant genotypes indicated by red arrows.

Crossing	No. of genotypes	Presence of marker ST1	Absence of marker ST1	Segregation ratio
01.607 x (Lady Rosetta x S440)	48	26	22	1:1
White Lady x W870	64	31	33	1:1
White Lady x 97.1011	30	15	15	1:1
White Lady x Latona	32	13	19	1:1

Table 2. Summary of crosses made and plants selected with PCR marker ST1.

Results and conclusion

Results of marker test on parental lines and varieties are shown in Table 1 and Figure 2. Recombinant events were detected in 2 PVY resistant varieties. Although based on pedigree data varieties Góliát and Chipke contain the Ry_{sto} gene, the ST1 marker could not be detected. This could be explained by the genetic distance between the gene and the marker or by presence of a different allele or locus, or by other source of extreme resistance.

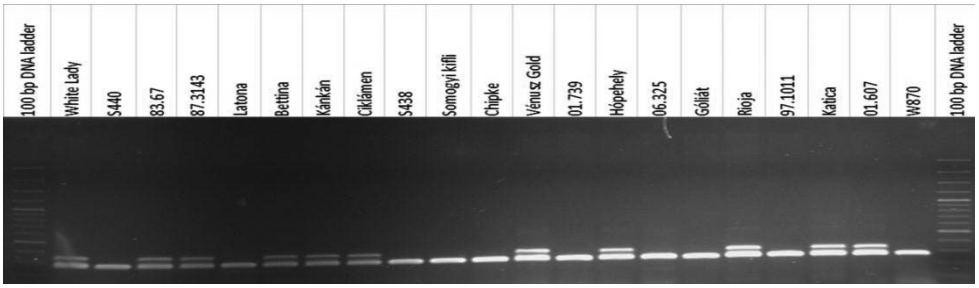

Figure 2. Amplification pattern of the ST1 marker in cultivars and breeding lines. Marker fragment is indicated by a yellow arrow. Recombinant genotypes are indicated by red arrows.

Totally, 174 F_1 genotypes from 4 different families were screened for the presence or absence of the marker ST1. Results are shown in Table 2. Based on PCR analysis the Ry_{sto} gene behaves as a single dominant gene and shows 1:1 segregation ratio. For final confirmation of the reliability of marker data the 174 F_1 genotypes will be exposed to natural PVY infection under field conditions and going to be tested by DAS-ELISA. At this stage of research we can conclude that the marker ST1 can be used for selection of PVY resistance in practical potato breeding with some limitations. Pedigree data of resistant parental lines must be confirmed for the presence of Ry_{sto} gene/ST1 marker before screening a segregation population with the marker.

The use of resistant cultivars is the best practical approach against the negative effects of virus infection in potato production all over the world. To facilitate the breeding of resistant cultivars the intensive use of molecular markers is a promising approach. However, effective and reliable molecular markers can only be developed on the basis of adequate molecular genetic information. Previous studies indicated that wide scale usability of Ry markers is very much dependent on the genetic background which the marker was developed (Cernák et al. 2008) from.

In our experiments 21 potato genotypes with known reaction to PVY and 4 segregating populations with 171 individuals were screened for the presence of ST1 marker linked to the gene Ry_{sto}. The practical usefulness of the ST1 marker for the identification of PVY resistant genotypes in a commercial breeding program was successfully proved.

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Durable Adult-Plant Resistance Gene Pb1 and Its Marker-Assisted Selection in Rice Breeding

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Abstract

Durable partial resistance to rice blast was found in some Japanese rice cultivars harbouring the rice stripe virus (RSV) resistance gene, *Stvb-i*, derived from an *indica* variety 'Modan'. These cultivars have not shown any breakdown of resistance to panicle blast for 30 years in Japan. A novel major gene, *Pb1*, conferred the "panicle blast resistance" in RSV-resistant cultivars. The *Pb1* locus was mapped in the Modan-derived region of chromosome 11, and map distance between *Pb1* and *Stvb-i* was 5.2 cM. Based on the linkage and graphical genotyping analyses revealed that RFLP marker S723 was the closest to *Pb1* gene among the tested markers. The *Pb1* gene does not confer any complete resistance with hypersensitive reaction, and the protective ability of the gene against panicle blast is sufficient for commercial rice production in Japan except in environments highly conducive to leaf blast. Since *Pb1* is considered to be a gene conferring durable adult-plant resistance, it is important in plant protection and its marker-assisted selection is quite useful in rice breeding.

Keywords: adult-plant resistance, durable resistance, partial resistance, rice blast (*Magnaporthe oryzae*), panicle blast, rice (*Oryza sativa*), major gene, marker-assisted selection (MAS).

1. Introduction

Rice blast (*Magnaporthe oryzae*) disease is one of the most widespread and destructive diseases of rice. This disease is classified into two forms: leaf blast and panicle blast. Panicle blast occurs after heading and directly reduces both yield and quality of rice grain. It consequently causes severe damage under blast-conducive environmental conditions. In Japan, most of the rice leading cultivars (e.g. 'Koshihikari'

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etc.) with excellent eating quality are susceptible to blast, so that they need fungicide application in agricultural production to control this disease. Therefore, growing resistant cultivars is an effective and ecological measure for controlling rice blast and for reducing the use of agricultural chemicals. However, complete resistance conferred by a major R-gene often breaks down within a few years after the dissemination of rice cultivars with R-gene [1]. This is caused by the occurrence of blast fungal race(s) that have adapted to the R-gene [2]. On the contrary, rice cultivars with partial resistance often show durable resistance to blast [3]. Partial resistance is a quantitative nature and usually conferred by several partial resistance genes [4]. Hence, it is not easy to incorporate several minor blast resistance genes into one elite cultivar by conventional breeding methods.

2. Discovery of a unique panicle blast resistance in RSV-resistant rice

Strong partial resistance to rice panicle blast was found in Japanese rice cultivars 'Tsikinohikari' and 'Asanohikari' harbouring the rice stripe virus (RSV) resistance gene, *Stvb-i*, derived from an *indica* variety 'Modan' [5]. We conducted a series of filed and laboratory studies to elucidate characteristics, durability, origin and genetics of the "panicle blast resistance" shown in RSV-resistant varieties. The experiments had been carried out in Nagakute and Toyota located in the central part of Japan for 11 years from 1984 to 1993 and 1996. The genotype of complete resistance to blast of 'Tsukinohikari', 'Asanohikari' and 'Aichi 67' were estimated to be *Pii*, *Pia Pii*, and *Pia*/+, respectively. All of them were common genotypes in Japan. Races 007 and 037 of the blast fungus were the prevalent races in the test field, and both of these races were virulent to all the varieties used in the test of blast resistance. Partial resistance to leaf blast of 'Tsukinohikari', 'Asanohikari' and 'Aichi 67' was comparatively low. 'Tsukinohikari' and 'Aichi 67' were found to exhibit a moderate to intermediate resistance to leaf blast, and 'Asanohikari' an intermediate resistance [6]. On the other hand, in the field, 'Tsukinohikari' and 'Aichi 67' were estimated to be highly resistant to resistant to panicle blast, while 'Asanohikari' were resistant [6]. Above results showed that the three RSV-resistant varieties expressed significantly stronger resistance to panicle blast than to leaf blast.

3. Durability of the panicle blast resistance

'Tsukinohikari' showed a durable resistance to panicle blast that persisted during an experimental period of 11 years in environments conducive to virulent blast races in both test fields in Toyota and Nagakute (Fig. 1) [6]. Furthermore, 'Tsukinohikari', its sister varieties and their progeny varieties (e.g. 'Matsuribare', 'Aichinokaori-SBL, 'Daichinokaze', 'Sainokagayaki', 'Yumematsuri', 'Koshihikari-Aichi-SBL', etc.) with RSV resistance have not shown any breakdown of their resistance to rice panicle blast for 30 years in farmers' paddy fields located in different areas of Japan from the dissemination of these varieties thus far [7]. Above results strongly suggest that these varieties with RSV resistance express durable resistance of a quantitative nature to rice panicle blast.

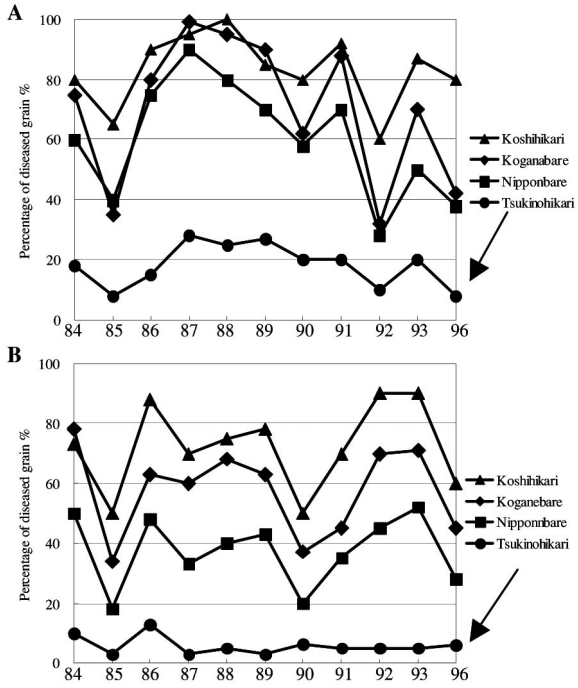

Fig. 1 Fluctuation of the percentage of blast-diseased grain of rice variety 'Tsukinohikari' in blast-conducive environments in Toyota (A) and Nagakute (B) for eleven years. (Reprint from Fujii et al. (1999), Breeding Reserch 1.)

4. Gene analysis of the panicle blast resistance of a quantitative nature

The experiments for panicle blast resistance had been carried out using the F_1, F_3 and F_4 lines in environments conducive to the occurrence of severe blast disease. These experiments resulted that the panicle blast resistance of a quantitative nature was considered to be a dominant character. Furthermore, the results of these experiments indicated that a novel major gene controls the panicle blast resistance of a quantitative nature. Therefore, Fujii et al. designated this novel resistance gene as *Pb1* (**P**anicle **b**last resistance-**1**) [8]. They also performed a linkage analysis between the panicle blast resistance gene *Pb1* and a RSV resistance gene *Stvb-i* using F_3 lines. The results showed that the two resistance genes are linked to each other with a recombination value of 5.2% [8].The *Stvb-i* gene has been mapped on the long arm of rice chromosome 11 [9]. The linkage relationship between *Pb1* and *Stvb-i* suggested that the *Pb1* locus is located near the *Stvb-i* locus on chromosome 11.

5. Genetic mapping and marker-assisted selection of the Pb1 gene

Fujii et al. precisely mapped the *Pb1* locus for the panicle blast resistance of a quantitative nature on rice chromosome 11 using restriction fragment length polymorphism (RFLP) markers [10]. Based on the linkage relationship between *Pb1*

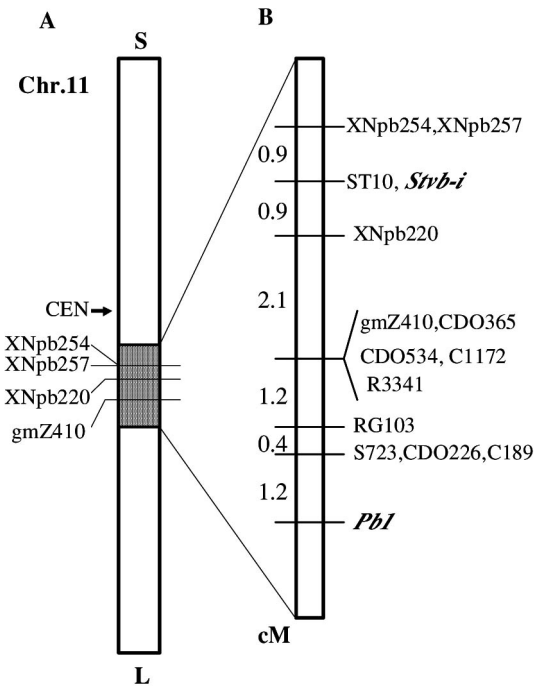

Fig. 2 RFLP map of the genomic region around Pb1 gene on rice chromosome 11 (B). The shaded region indicates the segment derived from var. 'Modan' (A). (Reprint from Fujii et al. (2000), Breeding Science 50.)

and *Stvb-i,* which derived from an indica variety 'Modan', they examined the linkage relationships between *Pb1* and 13 *Stvb-i*-linked RFLP markers located on the long arm of chromosome 11. As a result, *Pb1* was mapped in the 'Modan'-derived chromosomal region in the middle part of the long arm of chromosome 11 (Fig. 2) [10]. This result proved that both resistance genes, *Pb1* and *Stvb-i,* were incorporated into Japanese varieties from an *indica* landrace 'Modan' (Fig. 2). Linkage and graphical genotyping analyses revealed that RFLP marker S723 was the closest among the tested markers. Map distance between the two loci of *Pb1* and S723 was calculated to be 1.2 centimorgan (Fig. 2) [10]. Touyama et al. developed a couple of STS markers and a CAPS marker for practical marker-assisted selection (MAS) of *Pb1*, based on the genomic sequence in and around the cDNA clones S723 [11]. Consequently, 'Koshihikari-Aichi-SBL' was developed as the first near-isogenic resistant cultivar of 'Koshihikari' for *Pb1* and *Stvb-i* selected by MAS [12]. The eating quality of 'Koshihikari-Aichi-SBL' was as excellent as that of recurrent parent 'Koshihikari' [12].

6. Quantitative evaluation of the protective effect of the Pb1 gene

Evaluation test using four kinds of near-isogenic line (NIL) pairs revealed that protective effect of the *Pb1* gene against rice blast increased significantly with the progression of the growth stages of the rice plants; leaf (vegetative growth) stage <

Table 1 The protective effect of *Pb1* gene against rice blast increased with the progression of the growth stage of the rice plants.

Year	Place	Protective value of *Pb1* gene against rice blast[a]			n	Difference[b]
		Leaf stage	Flag-leaf stage	Panicle stage		
2000	Nagakute	48.3	-	94.7	12	46.4***
		68.1	-	95.2	16	27.1***
	Toyota	69.6	83.4	-	12	13.8***
		-	83.4	92.1	12	8.7**
		69.6	-	92.1	12	22.5***
2001	Nagakute	74.6	-	92.8	18	18.2***
	Toyota	73.4	-	93.0	12	19.6***
		77.2	-	93.3	24	16.1***

[a] Average of four near-isogenic line pairs for *Pb1* gene.
[b] *** and ** indicate $P < 0.001$ and $0.001 < P < 0.01$, respectively.
(Reprinted from Fujii et al. 2005, Breeding Research 7.)

Fig. 3 A typical symptom of rice panicle blast with neck and branch rot. Susceptible sib-line without Pb1 gene shows severe incidence of panicle blast (right). While, NIL with Pb1 gene confers strong panicle blast resistance of a quantitative nature (left).

flag leaf stage < panicle (reproductive growth) stage (Table 1) [13]. Therefore, *Pb1* gene conferred the adult-plant resistance to rice blast especially after heading. *Pb1* gene is a major gene, but does not confer any complete resistance with hypersensitive reactions, and the protective ability of *Pb1* gene against rice panicle blast is sufficient for commercial rice production in Japan, except in environments highly conducive to leaf blast (Fig. 3) [13]. Furthermore, *Pb1* gene showed a secondary protective effect on rice quality under the blast fungus-conducive environment [13].

7. Discussion on the durability of the Pb1 gene and concluding remarks

Although a major gene *Pb1* confers strong quantitative resistance to panicle-blast, the breakdown of this resistance has not been reported for 30 years since the beginning of commercial cultivation of rice cultivars with *Pb1* in all over Japan [7]. *Pb1* mediates adult-plant quantitative resistance to panicle blast, which is associated with increased expression of the gene in the adult stage. We speculated the durability of *Pb1* gene is closely related to its gene expression mechanism of adult-plant resistance. The selection pressure of *Pb1* gene to blast fungal population during leaf-blast stage should be significantly low compared with R-gene conferring complete resistance to leaf-blast, since *Pb1* gene only expresses intermediate or moderate resistance for leaf-blast and strong one for panicle-blast. This type of gene expression might avoid the occurrence and domination of blast fungal race(s) or lineage(s) that have adapted to the resistance gene in rice fields [6, 13].

Pb1 gene was recently isolated in 2010 by Hayashi et al [14]. *Pb1* gene was revealed to encode a CC-NBS-LRR protein. However, the *Pb1* protein sequence differed from previously reported R-protein, particularly in the NBS domain, in which the P-loop was apparently absent and some other motifs were degenerated [14]. *Pb1* transcript levels increased during the development of the cultivars with *Pb1*; this gene expression pattern accounts for the adult-plant resistance shown in rice cultivars with *Pb1* [14]. Since *Pb1* is considered to be a unique gene conferring durable adult-plant resistance, it is important in plant protection and its marker-assisted selection is quite useful in rice breeding. Further studies to reveal gene function of *Pb1* and gene-pathogen relationship have been conducting. Furthermore, gene-pyramiding breeding of *Pb1* and other partial blast resistance gene (e.g. *pi21*, *Pi39*) has been trying in some research stations in Japan. Consequently, positive effects of gene combination were observed between *Pb1* and *pi21* as well as *Pb1* and *Pi39* to control leaf blast and panicle blast [15, 16]. Hence, gene pyramiding of *Pb1* and other partial resistance gene(s) will enhance leaf blast resistance as well as reinforce stability and durability of blast resistance in rice.

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Localization of the common markers on the pea maps Wt10245 x Wt11238, Carneval x MP1401 and P665 x Messire (Pisum sativum L.)

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Key words: molecular markers, genetic mapping, QTL

Summary

Saturated genetic maps are necessary for QTL analyses, for a comparison of loci localization in different genetic backgrounds and for identification of molecular markers linked to some chosen genomic regions. Three maps were chosen; Polish Wt10245 x Wt11238, Canadian Carneval x MP1401 and Spanish P665 x Messire. Our first aim was the comparison of QTL localization on those maps. However, this was not possible because of a few common markers in these maps. We started to supplement the maps by new sequence-defined markers or other markers related to the reference *Pisum* map [1]. The previous version of the Wt10245 x Wt11238 map has already been published by Krajewski et al. [2]. Tar'an et al. [3] conducted the experiment, using the Canadian Carneval x MP1401 population, to identify the loci responsible for lodging resistance, plant height and resistance to mycosphaerella blight. Fondevilla et al. [4] detected several QTLs associated with resistance to *M. pinodes* using the Spanish P665 x Messire population. The sequence-defined markers came from the Grain Legumes Integrated Project. Nine markers were placed on the Wt10245 x Wt11238 map. Ten morphological and isozymic markers were analysed in the P665 x Messire population. Two isozymic and ten sequence-defined markers were studied in the Carneval x MP1401 population. The localization of these markers may be compared to the genetic map of *Medicago truncatula* and narrow-leafed lupin (*Lupinus angustifolius*) [5].

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Introduction.

Pea is one of a few legume species widely adapted to cultivation and also very useful as a model crop in genetics. The pea linkage map has been developed gradually during the past 50 years and improved versions of pea map have been published. The reference *Pisum* map was published by Weeden et al. [1]. Saturated genetic maps are necessary for QTL analyses, a comparison of their localization in different genetic backgrounds and identification of molecular markers linked to chosen genomic regions. The common markers localized on different maps are necessary.

The previous version of the Wt10245 x Wt11238 map was published by Krajewski et al. [2]. The map contained 191 markers of the length of 1086 cM and the average distance between markers of 11 cM.

Tar'an et al. [3] constructed the map for the Carneval x MP1401 population with 139 markers AFLP, 13 RAPD and 1 STS. The map length was 1 274 cM with 6,2 cM average distance between the markers. An improved version of the P665 x Messire map was published by Fondevilla et al. [6]. The inclusion of the SSR markers into this map resulted in a map containing 282 markers. The new map covered 1188.58 cM with an average inter-marker distance of 5.25 cM and contained the nine LGs.

The primer pairs were designed and synthesized within the Grain Legumes Integrated Project, the Sixth Framework Programme. The primer sequences were projected on single or low copy genes from *Medicago truncatula* or *Pisum sativum*. TC/EST sequences were chosen from the EMBL databases, proceeded and selected for primer designing to amplify legume consensus sequences with one or more introns not exceeding 2000 bp. SSR markers (Simple sequence repeats) were also analysed. They are useful in genetic mapping due to a broad range of polymorphism and the reading ease of electrophoretic pictures.

Material and methods.

The population of F2-derived lines was obtained from the cross Wt10245 × Wt11238 and described by Krajewski et al. [2]. The linkage map was constructed by the maximum likelihood method in JoinMap ver. 3.0. [7]. The mapping population Carneval x MP1401 was described by Tar'an et al. [3]. The P665 x Messire population of 111 F6:7 recombinant inbred lines was described by Fondevilla et al. [4]. An isozyme analysis was described by Wolko and Weeden [8].

The primer pairs were developed within the framework of the 6th EU FP Grain Legumes Integrated Project (GLIP). The primer information is available on the following website: http://bioweb.abc.hu/cgi-mt/pisprim/pisprim.pl. The SSR primer sequences and reaction conditions were published by Burstin et al. [9]. The comparison with *Medicago truncatula* sequences was done on the basis of *Medicago truncatula* HapMap project page http://www.medicagohapmap.org/?genome. The *Lotus japonicus* genome sequence version 2.5 was used to generate pseudomolecules representing the six chromosomes of *Lotus japonicus*. The pseudomolecules were assembled on the basis of the Kazusa clone lists (http://www.kazusa.or.jp/lotus/clonelist.html) using custom Perl scripts (http://www.perl.org/). A comparison of the pea genetic map and the genome sequence of *Lotus japonicus* was achieved via blastall BLASTn homology search.

Marker name	Pea linkage group	*Medicago truncatula*			*Lotus japonicus*			Lupin linkage group
		Annotation/clone name	Pseudo - chromosome	Closest marker/ position (cM)	Contig name	Clone name	Chromosome	
mtmt_EST_03569_02-03_1	I	AC153459, mth4-3g17	MtChr5	h2_14i8g /67.6 cM	-	LjSGA_011798	-	*L.ang* _LG9
UNK7	~ III additional	AC119412, CU326391, mth2-31d18	MtChr6	001A10 /1.4 cM	-	-	-	*L.ang* _LG18
psat_EST_00198_02_2	III	CT954236, mth2-3h12	MtChr3	h2_17f20a/61.6 cM	-	-	-	-
Pis_GEN_57_1-2_1	III	CU468574, mth2-69a16	MtChr3	h2_71p5a /6,3 cM	-	LjSGA_030827	-	-
mtmt_GEN_00069_05_1	IV	AC171266, mth2-14o4	MtChr8	004D01 /1,6 cM	-	LjSGA_116925.0.	-	-
mtmt_CON_03177_01-03_1	um. ~ V	CT962502, mth2-123f2	MtChr7	h2_20k24g /37,8 cM	-	-	-	*L.ang* _LG11
Pis_GEN_9_3_1	V	AC156627, mth2-168e8	MtChr4	004A06 /0 cM	CM2049.20.r2	LjT20B13	I	-
mtmt_GEN_00082_01_1	VI	AC146756, mth2-15l17	MtChr2	h2_15L17b /35,6 cM	-	-	-	-
Pis_GEN_16_2_1	VI	AC149581, mth2-126ml3	MtChr1	h2_49g13b /59,6 cM	CM0200	LjT35J07	V	-

Tab. 1. Comparative mapping of the Wt11238 x Wt10245 population – Medicago truncatula – Lotus japonicus – lupin.

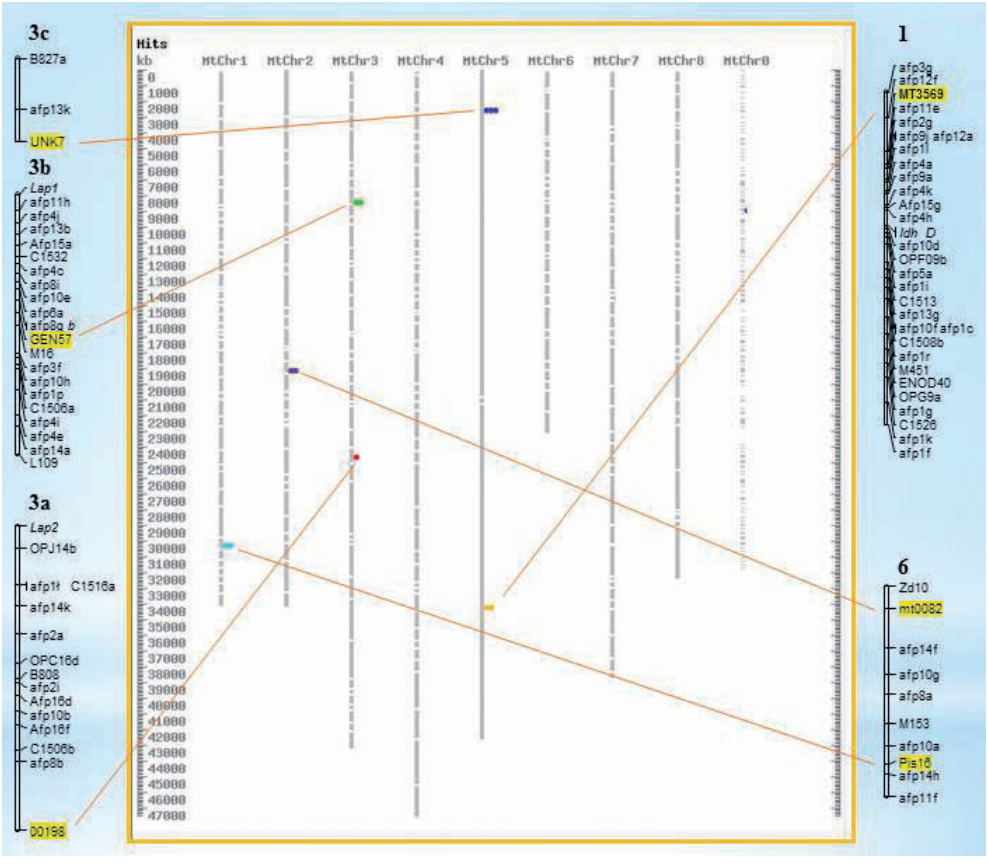

Fig. 1. Comparative mapping of the Wt11238 x Wt10245 population – Medicago truncatula.

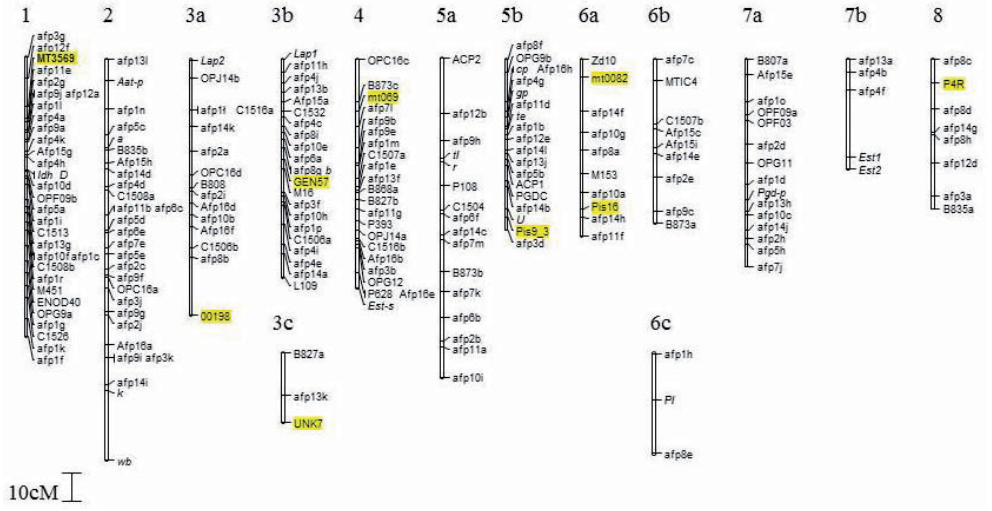

Fig. 2. The Polish linkage map with new markers.

Results.

The parental lines Wt10245 and Wt11238 were analysed using 103 primer pairs. Thirty three pairs were chosen due to the PCR product quality. The PCR products were sequenced and analysed using FinchTV www.geospiza.com/finchtv/ and Sequencher www.genecodes.com. *Nine sequence pairs were polymorphic. The analysis of the polymorphic sites was done using* dCAPS Finder 2.0 http://helix.wustl.edu/dcaps/dcaps.html. Nine markers were placed in the I, III, IV, V, VI and VIII linkage group. After the changes, the map contains 205 markers, the length is 944 cM and the average distance between markers is 6 cM. *We compared it with Medicago truncatula, Lotus japonicus and Lupinus angustifolius maps (Tab. 1, Fig. 1).*

There were no morphological markers in the Carneval x MP1401 population. Twenty one isozymic markers were tested in this population. Only two markers were polymorphic: Lap2, 6Pgd-p. The saturation with sequence-defined markers was described by Knopkiewicz et al. [10].

Seven monogenic morphological traits [11] were assessed in the P665 x Messire population in the following loci: *A*, *D*, *Pl, U*, *Rup* and *Sru*, *Le.* Twenty one isozymic markers were tested. Three isozymic markers were polymorphic in the population: *Aat-m, Est2, Idh*. One sequence-defined marker L109 was polymorphic in the parental lines P665 and Messire. There were five common markers for the Polish and Canadian populations (*Lap-2, 6Pgd-p*, UNK7, Pis_GEN_57_1_2_1, P628). The Spanish and Polish populations had 9 common markers (*A, D, Pl, U, Aat-2, Est-2, Idh*, P393, L109). There were no common markers for the Spanish and Canadian populations. The version of the Polish linkage map with new markers was presented in Fig. 2.

Conclusions.

The sequence-defined primers projected for Medicago truncatula are suitable for pea map construction. Polymorphism in the Polish population was higher than in the Canadian and Spanish populations (27%). Nine detected markers corresponded to sequences in the Medicago truncatula genome. Only 2 correspondences between the pea and Lotus japonicus genomes were found. Three markers were common for the pea and the lupin maps. There was no clear conserved collinearity on the linkage groups of pea and other analysed plant species. This provides an additional proof of a wide evolutionary distance between members of the Fabaceae family. The Carneval x MP1401 population was less polymorphic than the P665 x Messire population regarding the isozymic markers (10% versus 14%). The polymorphisms of sequence-defined markers was on the similar level, but the set of this markers is unlimited in comparison with isozymic markers. The investigation should be continued to obtain an appropriate number of markers to compare QTL localization for important agronomic traits and pathogen resistance.

Funding

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Molecular markers as the mechanism of fixing genes complex defining heterotic effect

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Each heterosis hybrid carries complex of genes, increasing productivity, though selected by breeder complex is unique in each hybrid. There is no big difference between heterosis hybrid and high productive variety. In both cases there are the same genes complexes worked. Problem of fixing heterotic complex of genes can be solved by means of elimination from genotype of hybrids lethal and half lethal gene, obtained from both parents and preserving at the same time complex of favorable genes. For this purposes we developed methodology, according of this method it is possible to release varieties with analogous yield on the basis of outstanding hybrid combinations in a short time.

Introduction. Hybrids not only exceed the yield of the best traditional varieties by an average 20–50%, but they have increased protein in grain and are highly adaptive to unfavorable environmental factors. However technologies of reception of hybrid seeds are very labour-consuming, that leads to their much higher cost (at rice in 10 times above). Other problem of hybrids production - necessity of their annual reception.

In the same time yield potential of newly-released varieties by conventional breeding method in various regions of the world remains at the same level many years. The reason is to collect a complex of the best genes increasing productivity in one sample during screening in late generations is unlikely occurrence. It is possible to overcome this barrier using achievement of heterosis breeding. Each heterosis hybrid carries complex of genes, increasing productivity, though selected by breeder complex is unique in each hybrid. There is no big difference between heterosis hybrid and high productive variety. In both cases there are the same genes complexes worked. In this connection several approaches have been suggested for maintaining the heterosis effect or in other words to fix a gene complex defining geterotic effect: the creation of apomictic hybrid, vegetative propagated plant and transferring genes responsible for the formation of the corresponding traits from other types of plants . In most cases for fastening geterotic effect in the subsequent generations it was offered to

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fix a hybrid genotype completely by translating a hybrid on sexless reproduction . But in the 1999 Strunnikov V. A. show, that for preservation of geterotic effect is not necessity to keep all genes complex of hybrid (1-2). Removal from their genotype lethal, semilethal or not effectively operating genes and in the same time preservation of a complex of favorable genes allows to achieve the same effect. Problem of fixing heterotic complex of genes can be solved by means of elimination from genotype of hybrids lethal and half lethal gene, obtained from both parents and preserving at the same time complex of favorable genes. Productivity of samples received on base of hybrid in this case not decrease. For removal of lethal and semilethal genes it was offered to use anther culture.

In pollen grain works about 30% genes of plant, consequently we can select best allels of 10000- 15 000 gens. In this case we work with big complex of genes wich determine viability of organisms. Frequency of survived individuals in anther culture does not exceed 0.5%. Individuals can survive, if it genotype has minimum quantity of lethal and maximum of favourable genes. The genotypes with lethal and halfhalf-lethal genes were eliminated, but a complex of favorable genes is preserved with survived plants. This allows us clearing genotype of initial hybrid from lethal and halfhalf-lethal genes, preserving at the same time complex of favorable genes.

Materials and Method. For this work we use some heterotic hybrid: Boiarin × Isumryd , Khazar Liman, Isumryd× Liman and population of double haploid line obtaining through anther culture on base of this hybrid. For analysis elimination loci from genotype of hybrids we use SSR molecular marker connected with adaptivity traits (cold, salt, drought tolerance). DNA extraction according to the CTAB method described by Murray and Thompson . The 100 SSR primers were used for polymorphism analysis of the parents. The polymorphic markers were then used for analysis double haploid line. DNA bands were visualized via ethidium bromide staining on 8% agarose gel.

Result. Problem of fixing heterotic complex of genes can be solved by means of elimination from genotype of hybrids lethal and half lethal gene, obtained from both parents and preserving at the same time complex of favorable genes. For removal of lethal and semilethal genes it was offered to use anther culture. For confirmation possibility using anther culture for removing lethal and half –lethal loci from hybrid genotype necessary to show selective elimination of allele from population of double haploid line from one combination Efficiency of using anther culture for this purpose is shown by us on molecular and organism level (3-5).

Studying of frequency of occurrence various allels of polymorphic markers in population double haploid lines has shown selective elimination of some alelles. As we expected the most part of markers for studied population was not selective. Or in other words distinctions by quantity of plants in population bearing allele of one and another parent were not statistically reliable (table 1), that speaks about equal probability of a survival of individuals with allels of both parents in anther culture. o Example of neutral for adaptivity allele in population of double haploid line, received on base of hybrid Boiarin × Isumryd we exposed using SSR marker RM 413.

However we reveal some allels that met in population not so often as other. Example of elective elimination of allele from population of double haploid line, received on base of hybrid Boiarin × Isumryd we exposed using SSR marker RM 164. Most of population of DH line has allele of pollinator.

Hybrid combination	SSR markers	Quantity of double haploid line with allele of first parent	Quantity of double haploid line with allele of second parent	χ^2
Boiarin × Isumryd	RM413	12	10	0,33*
	RM70	13	11	0,17*
	RM18	11	13	0,17*
	RM11	11	13	0,17*
Khazar Liman×	RM164	7	9	0,29*
	RM5688	11	6	1,47*
Isumryd× Liman	RM70	9	13	0,73*
	RM168	14	8	1,64*
* $\chi^2_{st} = 3,84$				

Table 1.-Markers, connected with adaptivity neutral allele

Hybrid combination	SSR-markers	Quantity of double haploid line with allele of first parent	Quantity of double haploid line with allele of second parent	χ^2
Boiarin × Isumryd	RM164	1	23	20,17*
Khazar Liman×	RM212	2	15	9,94+
Isumryd× Liman	RM164	22	1	19,17*
	RM5688	21	1	18,18*
	RM1	20	1	17,19*
P= 0.01(χ^2_{st} = 10,83); P = 0.05 (χ^2_{st} = 7,88).				

Table 2.- Markers , connected with selective elimination of allele

However at reception of doubled haploid in anther culture a complex of genes defining geterotic effect is distributed between various double haploid lines. To find lines bearing the maximum number of favorable genes in population doubled haploid lines does not represent complexity as all genes in homozygous condition. At a following stage it is necessary to unite the best genes of hybrid from various doubled haploid lines. For this purpose we use hybridization genetically contrast on the basis of molecular marking (SSR or SNP) the most productive doubled haploid lines of one hybrid combination.

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Mas breeding for BPH resistance in vietnamse rice

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Summary

Brown planthopper (BPH), *Nilaparvata lugens* Stal, which causes serious yield reduction by directly sucking the plants and acting as a vector of various diseases such as rice grassy stunt and ragged stunt, is one of the major insect pests of rice throughout the Asian rice – growing countries. Marker Assisted Selection (MAS) is a tool for enhancing the efficiency of Rice Molecular Breeding. Introgression of Bph3 and BphZ genes in the line IS1.2 into the elite cultivar SL12 was confirmed using MAS combine with conventional breeding. After generations, the most promissing rice line KR1(DTE2-3) was selected. The rice line KR1 was shown high resistance level with most of brown planthopper biotypes in Vietnam. Real revenue yield of the rice line KR1 was 5.5 to 8.2 ton/ha on the field since the year 2010 up to the first half year 2012.

I. Introduction

Rice (*Oryza sativa* L.) is an important cereal source for the world population as well as the main staple food for Vietnamese 86 millions people. The BPH is the most serious of the rice pests. It has caused devastating damage in China, Korea, Japan, and Vietnam... In 2005, China reported a loss of 2.7 millions tons of rice due to direct damage, while the loss reached 0.4 million tons in Vietnam, mainly by two virus diseases—grassy stunt and ragged stunt—carried by BPH in a persistent manner. Comprehensive information is available on the taxonomy of BPH outbreaks, migration, and varietal resistance, including chemical, biological, and cultural control (IRRI 1979) [1]. Several resistance genes were mapped and used for breeding seperatly. A total of 21 genes for BPH resistance have been identified from cultivated and wild species of *Oryza*. Of these 21 resistance genes, 15 are mapped to different chromosomal locations and 8 are tightly linked with molecular markers (Bra et al., 2009) [2]. Several breeding progammes were taken to develop new resistant varieties, but the fact is that, after few years, those varieties become succepible because

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of the biotypes changing. According to Jairin et al (2007) [3], *Bph1*, *bph2*, *Bph3*, and *bph4* have been used extensively in Thai breeding programs. Improved rice cultivars carrying *Bph1*, *bph2*, *Bph3*, and *bph4*, however, lost their ability against BPH, although cultivars with *Bph3* have shown a higher degree and broader spectrum of resistance against BPH. The development of biotechnology and molecular markers can allow us to speed up the rice breeding programs. Using MAS - Marker Assisted Seletion linked to Bph resistance genes not only improve the efficiency of selection but also reduce the breeding time. The main objective of this study was to develop 1-2 elite rice lines which resistant to BPH biotypes in Vietnam, high yielding and early maturing by molecular technology combine with conventional breeding method.

II. Materials and methods

The materials consisted of an elite breeding rice line SL12, later maturing, high yield, was used as donor recurrent cultivar. The line IS1.2 carrying Bph3 and BphZ resistance genes, highly resitant to BPH, early maturing, was used as donnor. The parents and additional cultivars, TN1 and PTB33, were used in experiments for evaluating BPH resistance in seedling, vegetative and reproductive stages of rice plants. Five Bph biotype sources, colected from provinces in Vietnam: Ha Noi, Nam Dinh, Nghe An, Dong Thap, Can Tho, were used to test the resistance ability of the promissing selected lines. The SSR markers linked to resistance genes Bph3 and BphZ were used to select the F2 to F6 and BC1 to BC4 plants carrying the resistance genes. Traditional breeding were also applied in paralell with marker assisted selection. Our aim was to find out the promissing line with good agronomic characters, early maturing and stable resisstance to BPH.

III. Results

In this study, BphZ is one of the major resistance gene from the cultival GC9, which have been mapped to the long arm of chromosome 4 when analysing the F2 pupolation of TN1xGC9 (Huyen et al., 2010) [4]. Bph 3, an dominant resistance gene from the cultival Rathu Heennati, mapped to the short arm of chromosome 6 (Jairin et al (2007) [3]. These two genes (Bph3 and BphZ) were pyramided into the line IS1.2 having two resistance genes but nearer to the domestic plant type. After that step, the genes in line IS1.2 were introgressed into the cultivar SL12. Base on the map of BphZ and Bph3 resistance genes, parental polymorphism were carried out on 14 and 7 markers distributed on both sides of the gene BphZ and Bph3, respectively. Of which, 7 markers RM3524; RM1388; RM3367; RM3735; RM5757; RM6997 were polymorphic for the gene BphZ and 4 markers RM586; RM588; RM589; RM190 were polymorphic for the gene Bph3. Those markers were used to select the promising lines having the two genes in every breeding populations from F2 to F6 and BC1F1 to BC4F2.

In figure 1, total 11 plants having band of resistant parent was confirmed the present of BphZ gene, and continued checking with the Bph3 gene. Of which, only 6 lines having the band of marker link to Bph3 gene, they were plants number 1, 2, 3, 7, 8 and 10. From this result, checking the resistant reaction in every generation combine with conventional breeding, one highly promising line was chosen namely KR1.

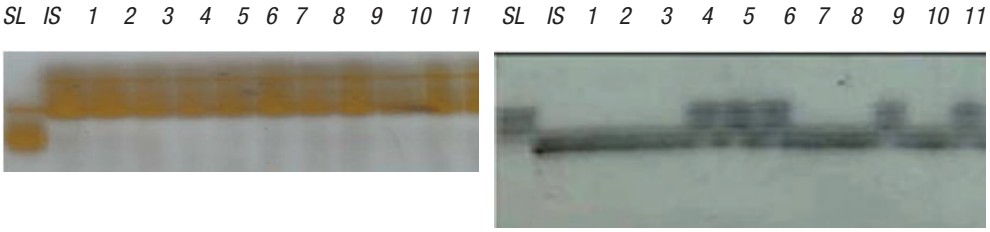

Figure 1. Using marker RM5757 link to BphZ gene to select the line having resistantce gene (left). Lane 1: SL12, Lane 2: IS1.2, Lanes 1 to 11: selected BC1F1 plant having band of IS1.2; Using marker RM588 link to Bph3 gene to select the line having resistance genes (right). Lane 1: SL12, Lane 2: SL12, Lane 3-13: selected BC1F1 plant having of IS1.2

N.o	Variety	Ha Noi	Nam Dinh	Nghe An	Dong Thap	Can Tho
1	**KR1**	**2**	**3**	**2**	**2**	**4**
2	IS1.2	2	2	2	2	2
3	Ptb 33	2	2	2	2	4
4	TN1	9	9	9	9	9

Table 1: Resistantce score at seedling stage of the promising line KR1 with biotypes of BPH colecting from difference provinces in Viet Nam

N.	Variety	Maturity time in Summer-Autunm seasson 2011 (day)	Maturiry time in Winter-Spring season 2012 (day)	Plant Height (cm)	BPH score
1	KR1	105 - 108	130 - 135	95 - 100	2
2	SL12	125 - 128	150 - 155	115 -120	8
3	IS1.2	105 -110	135 - 140	135 - 140	2

Table 2. Some of the agronomic traits of KR1 compare to parent varieties

N.	Variety	Ear/plant	Plants /m2	Seeds/ear	Filled grain (%)	P1000 (g)	Theory yield (t/ha)	Real yield (t/ha)
1	**KR1**	**6.37±0.67**	**50**	**195.33±7.8**	**92.90**	**22.7**	**12.9**	**8.2**
2	SL12	5.67±0.56	50	181.33±7.8	88.22	24.6	11.1	7.4
3	IS1.2	6.25±1.1	50	98.5±4.9	86.47	23.3	6.2	4.7
4	KD18	7.1±0.75	50	179.9±7.8	77.65	19.6	9.7	4.6

Table 3: Factors contribute to the yield of KR1 compare to parent and popular cultivar (KD18) in Winter-Spring season 2012

Figure 4: Reaction of F2 plant to BPH in the damage region Long An - Vietnam - 2007

Figure 5: The promising line KR1 at Nam Dinh – Vietnam – 2012 having the yield 8.2 ton/ha

Evaluating the reaction to brown planthopper of the promising line KR1 shown that this line highly resistant to the biotypes of BPH in Vietnam from the North to the South. The promising line KR1 was tested for VCU and DUS on the National testing Center in Vietnam.

In table 2, the promising line was put in the group of early maturity and pets resistant cultivars. In table 3, when compare KR1 to its parent and popular cultivar in Vietnam (KD18), the line KR1 showing its promising ability of high yield on the field.

Additionally, the KR1 was tested the resistance ability compare to some imported BPH and WPH resistant varieties from Japan in both vegetative and reproductive stage in NamDinh province. The results obtained were the KR1 stayed the highest resistance compare to all the others.

Figure 4 and figure 5 were illustrated for the reaction to BPH and potential of promising line KR1.

IV. Conclusions

Application of molecular marker in rice breeding for BPH resistance was shown highly effective when introgressing two BPH resistance genes together into an elite background cultivar. This study was successfull in developing the promising line KR1 having BphZ and Bph3 resisstance genes, early maturity and high yield and stable resistance to BPH. This KR1 variety was meet the demand for BPH resistance rice breeding in Vietnam.

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Low Cost Mutation Discovery Methods Suitable for Developing Countries

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Abstract

Mutation detection using the reverse-genetic strategy TILLING has been widely used for the evaluation of mutant plant populations and to target mutations in genes controlling specific traits. The most common method of screening for mutations requires the use of labelled primers and a fluorescence-based genetic analyzer. While the technique is sensitive and high-throughput, development of lower cost and reliable methods would be beneficial to laboratories with limited resources, such as in developing countries without access to specialized equipment. We and other groups have modified screening methods that utilize enzymatic mismatch cleavage and standard electrophoresis platform that it is suitable for the detection of induced and natural mutations in a range of various seed and vegetatively propagated crop plants.

Keywords: low cost TILLING/EcoTILLING, mutation discovery, SNPs

1. Introduction

TILLING (Targeting Induced Local Lesions IN Genomes) and EcoTILLING are high-throughput strategies for the discovery of induced and spontaneous point mutations in plant and animal genomes. The most common polymorphism discovery method applied to date uses fluorescently labelled PCR primers to amplify a gene region of interest. In the subsequent step, heteroduplexes in the amplification product are digested with the mismatch-specific endonuclease, CEL1. The digested PCR reactions are commonly visualised through laser detection of fluorescently labelled DNA fragments using equipment such as a LI-COR analyser. There are a number of applications and crops where the method was successfully applied for both mutation

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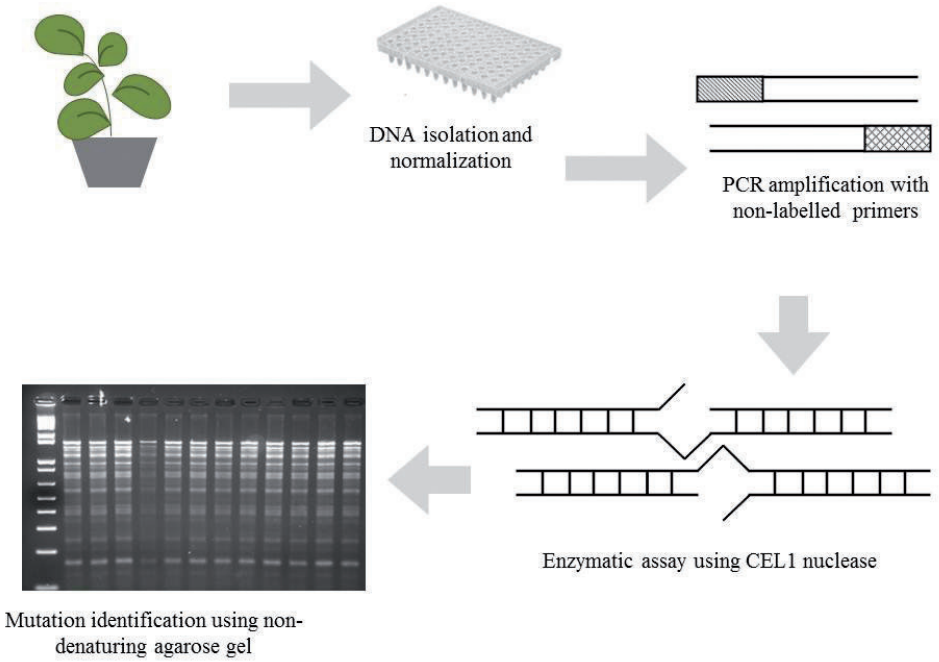

Figure 1; Simplified schematic representing a low cost agarose gel based TILLING or EcoTILLING procedure. DNA extracted from studied plants is amplified with the use of non-labelled PCR primers targeting genes of interest. The enzymatic heteroduplex digestion is applied in order to induce double strand breaks at the point of mismatch following the low cost protocol [8] and the product of CEL1 digestion is visualised on a 2% agarose gel composed of routine and fine agarose in a 1:1 proportion. Bands are separated during 2-3 hours run at 80 - 100V (depending on the product length) and visualized using GelDoc system (Bio-Rad). The gel represents a subset of a population of gamma induced banana mutants (cv. Gingeli) screened for the presence of unique mutations in the gene coding the GDSL-motif lipase/hydrolase protein. Only naturally occurring polymorphisms have been scored in this screened gene.

and natural polymorphism discovery [1]. A major drawback with higher throughput discovery methods is the reliance on specialised equipment and consumables limiting use to laboratories with sufficient infrastructure and operational budget. Adaptation of low cost strategies enables the transfer of technologies to laboratories with standard equipment such as agarose or polyacrylamide gel electrophoresis systems. This has led groups to develop alternative and cheap methods to replace costly genotyping and mutation discovery platforms ([2], [3], [4], [5], [6]). At the Plant Breeding and Genetics Laboratory (PBGL) we have undertaken studies to compare sensitivity and accuracy of lower cost and lower throughput *versus* high-throughput assays in a range of crop plants grown in developing countries. Since the basic molecular biology laboratory typically has standard capacity including horizontal electrophoresis and PCR, the objectives and research activities were concentrated on the usage of a simple agarose and non-denaturing polyacrylamide gel systems to detect digested non-fluorescently labelled fragments. This has been applied to both natural and mutated populations.

CROP	DETECTION METHODS	REFERENCE[a]
Arabidopsis	LiCOR, agarose gel	[8]
Rice	LiCOR, agarose gel, polyacrylamide gel	PBGL unpublished[b]; [5], [4]
Banana	LiCOR, agarose gel	[9], [10], [11]
Lupin	LiCOR, agarose gel	[12]
Tomato	LiCOR, agarose gel	PBGL unpublished[c]
Barley	agarose gel	PBGL unpublished[d]
Olive	LiCOR, agarose gel	PBGL unpublished[e]
Hexaploid wheat	polyacrylamide gel, agarose gel	[6], [3]
Tetraploid wheat	polyacrylamide gel	[6]

[a] Unpublished results are available upon request.
[b] Work carried out by Prof. Hairui Cui from China during the training fellowship.
[c] Work carried out by Ms Banumaty Saraye from Mauritius during the training fellowship.
[d] Work carried out by Ms Wahiba Amri Epse Tiliouine from Algeria during the training fellowship.
[e] Work carried out by Ms Raouia Dhouibi from Tunisia during the training fellowship.

Table 1; Summary of low cost projects developed by PBGL as well as by other research groups.

2. Methodology

Plant material used in these assays was extracted from: arabidopsis, rice, banana, lupin, tomato, barley, and olive with the use of DNA Qiagen kit (Qiagen). All the procedures for primer design and enzyme purification were applied following the standard TILLING protocol [7]. The low cost method for heteroduplex generation, digestion and separation of digested PCR products on agarose gels were optimized and applied (Fig.1; [8]). The optimization steps included: a) selection of DNA concentration; b) adjustment of PCR mix composition; c) identification of most effective concentration of CEL1 enzyme (using a crude extract prepared from celery); d) post-digestion purification; e) composition and concentration of agarose gels (usage of routine and fine agarose, concentration tested ranging from 1.5 - 5%) as well as band separation conditions and gel visualization.

3. Results

Working with scientists from developing countries the PBGL has successfully adapted low cost methods for a wide range of species (Table 1).

Digested PCR products were visualized on agarose or non-denaturing polyacrylamide gels and, as a control, run additionally on the LI-COR genetic analyzer. While band resolution is highest on denaturing PAGE LI-COR gels, the low-input alternative using agarose was able to detect sufficient heterozygous SNPs in the studied crops, and as shown in the example below where low cost methods were applied to accessions of tomato it was also able to differentiate samples accurately (Fig. 2).

In the case of complex genomes, such as banana, polyploidy and the frequency of natural heterozygous polymorphisms may impact on the accuracy of the low cost platform because of poor resolution of closely positioned SNPs in agarose gels. This makes genotyping of closely related natural banana accessions difficult. However the assay shows enough sensitivity to allow fast and easy distinction and classification of

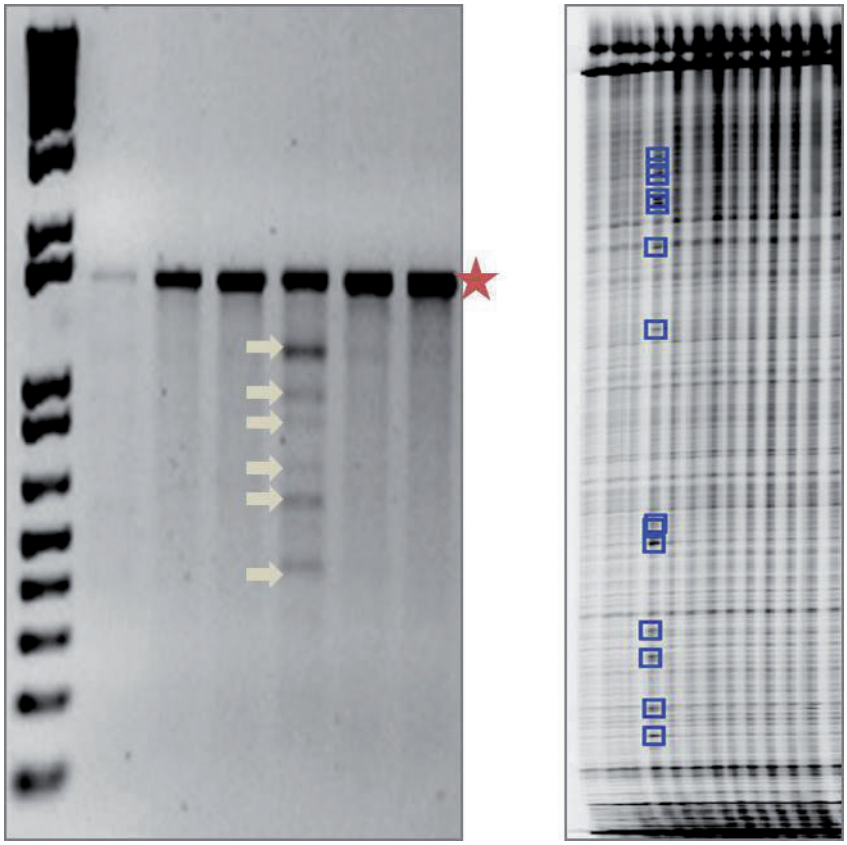

Figure 2; Comparison of low cost agarose gel electrophoresis and high-throughput LI-COR gels. Both gels represent screening of natural and induced point mutations in a tomato population. The left gel is a low cost agarose gel, whereas the picture to the right shows the LI-COR generated denaturing PAGE gel. Visible heterozygous bands appearing in both gels appear in the variety Metis, which showed significant differentiating from other tomato accessions.

different groups such as diploids, triploids, tetraploids and hybrids. Similar problems with band resolution in the case of complex genomes have been observed when using other electrophoresis platforms including higher resolution PAGE gels ([13], [10]). Thus, the optimal gel system is decided by factors including heterozygosity and the band resolution required to address the research question. In many cases agarose gels offer a cheap and time effective alternative method for quick discrimination among various plant accessions, even in highly heterozygous accessions. One useful application of low cost polymorphism discovery is for TILLING assays where the method could be applied as the primary TILLING screen or could be used as a genotyping method enabling tracking of identified and selected mutant plants. As we have shown in the case of banana mutant population (Fig.1), the agarose gel represents an ideal cheap alternative for quick detection of putative mutations. In this case, only individuals found to be different from control and from the rest of population may be selected for further screening.

4. Conclusions

From the different experiments described here, it can be concluded that simple, low cost TILLING and EcoTILLING can be used to assess both natural and induced polymorphism in various crop species. The methods are robust and do not require sophisticated instrumentation or costly labelled primers. It is also easily transferred to different plant species. This is especially important and useful for developing countries where there is often a lack of sophisticated molecular biology equipment. Where genomic sequence is available, but markers have yet to be developed, low cost EcoTILLING provides a means to capture SNP diversity and develop markers quickly. This is particularly helpful in detecting useful mutants.

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Application of Marker Assisted Backcrossing (MABC) to improve Salinity and Submergence Tolerance in BacThom 7

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Abstract: Vietnam is one of the most vulnerable countries to climate change in Asia. Rice is a principle food in Vietnam. However, rice yield and its cultivating areas are adversely affected by the threats of devastation caused by the rise of sea level and flash flooding. Using marker assisted backcrossing (MABC) to improve salinity and submergence tolerance in rice is one of the feasible methods to cope with these devastating changes. To improve rice salinity and submergence tolerance in BacThom 7, FL478 and IR64-*Sub1* cultivars were used as donor parents to introgress separately the QTLs *Saltol* and *Sub1* conferring salinity and submergence tolerance into BacThom 7. Three backcrosses were conducted to transfer positive alleles of QTL *Saltol* from FL478 into BacThom 7. The plants number IL-30 and IL-32 in BC_3F_1 population expected recurrent genome recovery of up to 99.2% and 100%, respectively. For submergence tolerance, a selected plant number IL-19 in BC_2F_1 population which carrying QTL *Sub1* has genetic background up to 89.8%. The success of improving rice of submergence and salinity tolerance by applying MABC could accelerate the development of superior qualities in the genetic background of BacThom 7.

Keywords: Background selection, foreground selection, marker assisted backcross, rice, QTL

1. Introduction

Increases in salinity and submergence resulting from climate change are the major impediments to enhancing production in rice growing areas worldwide. One-fifth of irrigated arable lands in the world have adversely influenced by salinity [1] and 15 million hectares of rainfed lowland rice areas in South and Southeast Asia are regularly

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affected by submergence stress [2]. Vietnam is one of the countries hardest hit by climate changes in Asia. By the end of the 21st century, temperature would rise by about 2.3^{o}C and sea level rise by 75 cm relative to the average of 1980-1999. Vast portions of the food producing regions in the country will be inundated by sea water, expected at about 19-37.8% of the Mekong River Delta (MRD) and about 1.5-11.2% of the Red River Delta (RRD). With sea level rise by 1 m, approximately 40,000 km2 will be inundated, and salinity intrusion is expected to cover about 71% of the MRD and RRD, together with other coastal regions [3,4]. Rice is the most important food crop for over half of the world's population and supplies 20% of daily calories. Rice is a major crop in Vietnam, as the world's second-largest rice exporter after Thailand, and together accounting for 50% of the world rice trade. However, rice yield and its cultivating areas are adversely affected by the threats of devastation caused by the rise of sea level and flash flooding which are causing significantly economic loss in this country. Therefore, the need for enhancement in salinity and submergence tolerance in rice is urgent work in this country to cope with climate change. Marker Assisted Backcrossing (MABC) is an accurate and effective method to introgress a single locus controlling a trait of interest while retaining the essential characteristics of the recurrent parents [5]. Recent studies conducted at International Rice Research Institute (IRRI) have been successfully transferred QTLs *Saltol* and *Sub1* into some modern rice varieties. The main objective of our study was to improve submergence and salinity tolerance of BacThom 7, a widely grown rice cultivar by using MABC method. The improved cultivar may be useful for growing in the soil salinity of the coastal areas and submergence prone areas of Vietnamese Deltas.

2. Materials and Methods

2.1. Plant materials and crossing scheme

The MABC scheme for constructing the plant materials used in this study is summarized in Figure 1. The highly salt tolerant FL478 (IR 66946-3R-178-1-1) and IR64-*Sub1* were used as the donors of *Saltol* and *Sub1* QTLs, and whereas BacThom 7 (*O. sativa spp. indica*), a popular growing Vietnamese elite cultivar with high quality was used as the recipient parent. DNA was extracted from juvenile leaves of 2-week-old plants using a modified protocol as described by Zheng et al. (1995) [6].

2.1.1.Foreground and recombinant selection

At the initial stages of the experiment, for selection of the *Saltol* locus (foreground), the reported rice microsatellite (RM) markers RM493 and RM3412b, which were found to be tightly linked to *Saltol* was used for foreground selection. For franking markers used for recombinant selection, about 5 Mb region of the *Saltol* region was targeted. Four polymophic microsatellite markers (RM1287, RM10694, RM562, RM7075) were identified for recombinant selection. Similarily, ART5 and RM23877 markers were used for foreground of sub1 locus and RM23662, RM5688, S09026B and RM24013 markers for recombinant selection.

2.1.2.Background selection

Microsatellite markers unlinked to *Saltol* covering all the chromosomes including

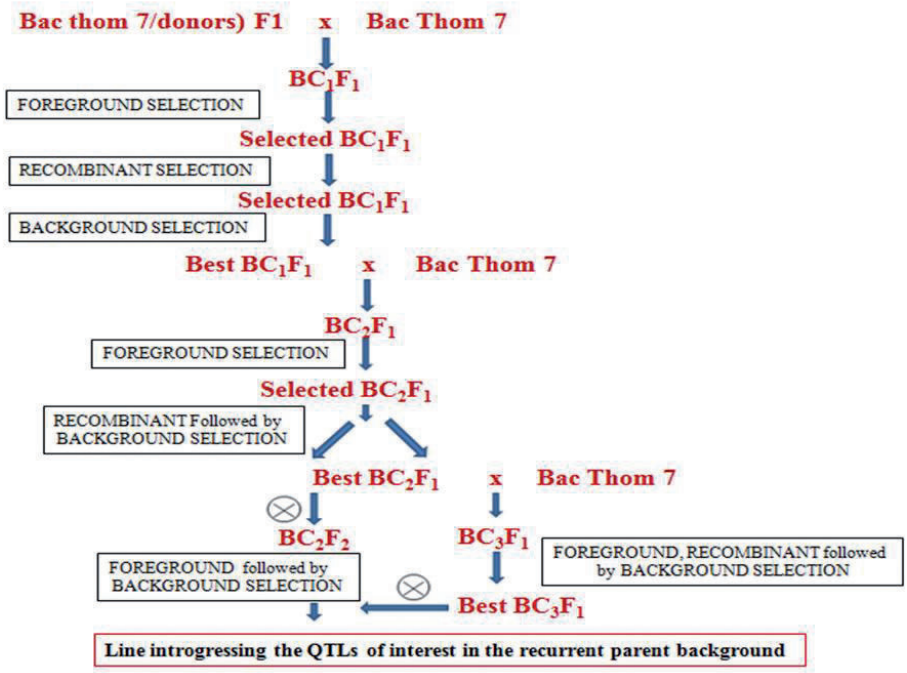

Figure 1: The scheme of applying MABC to improve salinity and submergence in BacThom 7

the *Saltol and Sub1* carrier chromosome 1 and 9, that were polymorphic between the two parents, were used for background selection to recover the recipient genome. Based on the polymorphic information, initially evenly spaced microsatellite markers were selected per chromosome. At least four polymorphic microsatellite markers per chromosome were used. The microsatellite markers that revealed fixed (homozygous) alleles at nontarget loci at one generation were not screened at the next BC generation. Only those markers that were not fixed for the recurrent parent allele were analyzed in the following generations. For the selected plants from BC_2F_1 and BC_3F_1, an additional 84 microsatellite markers were used to check the fixation of the recipient genome. The molecular weights of the different alleles were calculated by Alpha Ease Fc 5.0 software. The marker data was analyzed using the software Graphical Genotyper [7].

3. Results

3.1.Foreground and recombinant selections

As the obtained result from screening of 30 SSR markers at the target region on chromosome 1 for polymorphic markers, ten markers showed polymorphism between

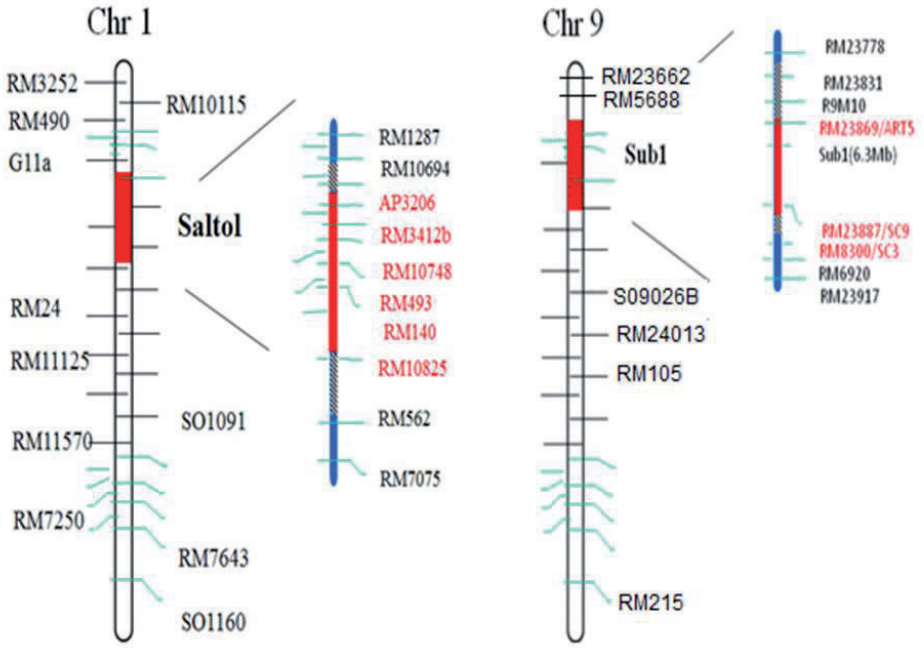

Figure 2: Graphical representation of the regions on chromosome 1 and 9 containing Saltol and Sub1 . White portions of the bar = homozygous Bac Thom segment, black regions = homozygous Saltol segment, and diagonal slashes = regions where crossing over occurred. Markers polymorphic between Bac Thom and FL478 are label on both sides of the chromosome. The estimated distances in kb between the SSR markers and their orders are available at www.gramene.org [8]

the parents. Two markers, namely RM493 and RM3412b tightly linked to *Saltol* and four markers RM1287 RM562, RM3252, RM490 were detected for foreground and recombinant selection, respectively.

In each backcross generation (BC_1F_1-BC_3F_1), the target locus *Saltol* was monitored by markers linked to the *Saltol* genes. However, only a few such selected individuals that had the least donor alleles of the background markers were chosen to be backcrossed with BacThom 7. In advanced backcrosses and selfed generations, marker polymorphic RM493 and RM3412b tightly linked with *Saltol* was used to screen. Four polymorphic markers between BacThom 7 and FL478 at target region were used to screen individual BC_1F_1 plants. In conjunction with background section, the *Saltol* carrier chromosome 1 of a few selected individuals, including plants number 1, 7, 8 and 26 in BC_2F_1, whereas, the plants numbers 10, 14, 30, 41, 359 in BC_3F_1 was characterized with two markers for foreground selection (RM493 and RM3412b). When the selected plants of BC_3F_1 (plants No. 10, 30, 32 and 359) were screened with these two markers, the alleles of markers from RM3412 (12597139bp) through RM493 (13376867bp) were of the donor (FL478) type, and the alleles of all the remaining markers from RM1287 (11836436 bp) to RM562 (16232926 bp) onwards were of BacThom 7, indicating that these plants were single recombinants (Fig.2).

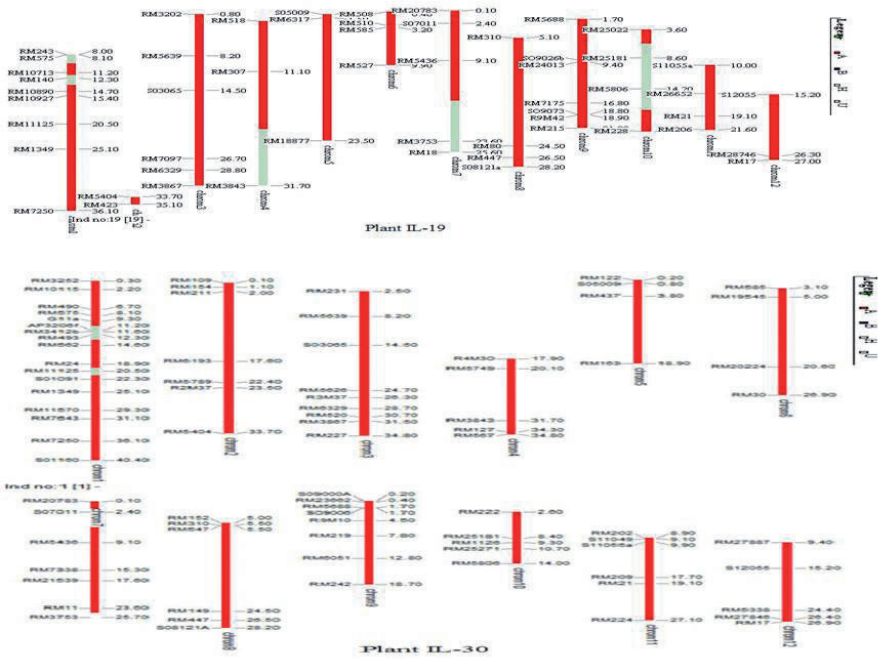

Figure 3: Graphical representation of the plant IL- 19 and 30 genotype. Chromosome numbers are located at the top of the bars. Black portions of the bars are derived from BacThom 7 and slash regions indicated the Saltol and FL478; Sub1 and IR64-Sub1 introgressions. Markers are labeled on the right side of the chromosomes.

For submergence, 11 out of 24 markers at the target region on the chromosome 9 showed polymophics between BacThom7 and IR64-*Sub1*, of which 6 markers namely RM23662, RM5688, ART5, RM23877, S09026B and RM24013 showed tight linkage to the region of *Sub1* (Fig. 2)

3.2.Background selection

A total of 477 SSR markers were screened for polymorphism between BacThom 7 and FL478 distributed on 12 chromosomes. Among them, 89 (18.7 %) markers showed polymorphisms between the parents and were used for background selection. In BC_1F_1,generation, a total of 30 markers was used for background selection in 25 BC_1F_1 plants. Based on the foreground and background selection, the two selected BC_1F1 plants (Nos. 7 and 13) were developed BC_2F_1 populations. In the BC_2F_1 population, 43 polymorphic markers were used for background selection in 19 BC_2F_1 plants resulting from foreground and recombinant selection plants No. 21, 41. For plant No. 21, chromosomes 5, and 8 were of complete recipient types. In this experiment, the background analysis of BC_3F_1 revealed the recurrent genome recovery of up to 100% at which individual lines were ranging from 81% to 100%. Specifically, the

recurrent genome recovered in the plants No.s IL-30, IL-32 is expected to be 99.2% and 100% , respectively. Similarly, for submergence, 58 out of 378 markers showed polymorphism between the parents distributed on 12 chromosomes. The *Sub1* carrier of individual plants in BC_1F_1 and BC_2F_1 generations were identified by 4 tight linkage markers namely ART5, SC3 and ART5, RM23877, respectively. Based on background analysis of BC_2F_1 population, the plant number IL-19 which carrying QTL *Sub1* has showed genetic background up to 89.8% (Fig. 3).

4. Discussion

Climate change is causing negative impacts on rice production in Vietnam. Most of area deltas where cultivate rice are already being affected by the rising sea level and flash flood, increasing the incidences of salinity and submergence in rice field. Hence, it is imperative to develop submergence and salinity tolerance of rice cultivars with acceptable yield potential by using modern tools of biotechnology. However, It is also challenging to achieve a definite goal of salt and submergence tolerance using conventional breeding strategies when the target gene is linked with an unfavorable dominant gene [9]. Nevertheless, using the tools of biotechnology, it is plausible to transfer valuable genes of salt and submergence tolerance stresses in rice without linkage drag [10]. In this study, BacThom 7 was selected as the recipient parent because it is good quality rice and always gives high profit for milled rice in Vietnam. Our study focuses on combining the useful agronomic traits of BacThom 7 with QTLs *Saltol* and *Sub1*, which attached salt tolerance in backcross breeding lines by conversion to the recurrent parent genotype using molecular genotyping with SSR markers. We have successfully transferred the *Saltol* and *Sub1* from donor lines FL478 and IR64-*Sub1* into BacThom 7. The *Saltol* and *Sub1* genes were identified in an introgression lines, highly salt tolerant FL478 (IR 66946-3R-178-1-1) and IR64-*Sub1* (IR40931-33) which inherited the gene from the Pokkali and FR13 A [11].

In this study, we used the MABC breeding method to transfer the *Saltol* and *Sub1* genes into a Vietnamese popular cultivar by phenotype and genotype selection. Using SSR markers (RM493, RM3412b) for the *Saltol* and (RM23662, RM5688, ART5, SC3 and RM23877) for *Sub1* genes ensured efficient foreground selection. The co-dominant nature of SSR markers could be very useful in addition to gene-based markers for the introgression of the *Saltol* and *Sub1* locus into a wide range of recipient elite cultivars. The selfed progenies or recombinant homozygote plants in the target region were selected from 300 to 478 plants for each backcrossing genenration with foreground selection. Our results demonstrated that the major salt and submergence tolerance genes (*Saltol* and *Sub1*) from the donor parents FL478 and IR64-*Sub1* have successfully transferred into the BacThom 7

5.Conclusions

We have successfully improved a salinity and submergence tolerance of BacThom 7 cultivars by using MABC method, which were controlled by the major *Saltol* and *Sub1* QTLs. The recovery of the recurrent parent genome by molecular genotyping and selection could increase the efficiency of the MABC, and this was achievable in a short span of time in rice breeding strategy. This study could have an good impact

in rice breeding and it is applicable for the introduction of important agronomic traits into the genomes of popular rice cultivars.

Acknowledgements

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Natural Variation in CBF Gene Sequence and Freezing Tolerance in Eucalyptus gunnii

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Abstract

Eucalyptus, considered as the most widely planted hardwood in the world, is an evergreen tree without endodormancy and consequently exposed to cold. CBF transcription factors are involved in the regulation of the cold response. The study of the existing variability in the CBF sequence in *E. gunnii* and that of the cold tolerance were performed by phenotyping and genotyping a set of plants. After clustering polymorphisms into haplotypes, sequence variations likely to affect gene regulation or protein activity were identified.

Introduction

Native to Australia, *Eucalyptus* species are the most widely planted hardwood trees in the world and among the fastest growing ones (FAO., 2005). The expansion of *Eucalyptus* plantations throughout the world is largely attributable to the increased global demand for short-fiber pulp. In addition, short rotation *Eucalyptus* plantations are important for replacing the use of fossil hydrocarbons for energy and industrial organic chemicals.

Despite of their very high attractiveness for industrial purposes, plantations are still limited to climatic regions without sever frosts. Thereby, freezing temperatures limit the geographic distribution of plants and reduce crop productivity and quality. Among more than 700 identified species, *E. gunnii*, exclusively originated from the Tasmanian mountains is one of the most cold tolerant but does not survive below -18°C. Actually, the physiology of this tree looks more dedicated to fast growth than adaptation to a stressful environment. It lacks for example seasonal leaf-fall, endodormancy and bud insulation. Due to the resulting strong exposure to winter frost, this freezing tolerant species appears like an interesting model to dissect cold tolerance.

Studies in *Arabidopsis* and other dicots, including *E. gunnii*, have demonstrated that CBF/DREB (C-repeat Binding Factor/Dehydration Responsive Element Binding)

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genes act as key regulators of plant cold tolerance and other stress responses. The CBF regulon represents up to 12% of the cold regulated genes in *Arabidopsis* (Cook *et al.*, 2004; Fowler *et al.*, 2005). To date, the DREB1/CBF signaling pathway has been one of the most explored for improving crop stress tolerance (Thomashow, 2010; Mizoi *et al.*, 2011). *In E. gunnii, 4 EguCBF1 genes are differentially regulated under* different cold stress conditions (El Kayal *et al.*, 2006; Navarro *et al.*, 2009)**.** Moreover, the overexpression of two of them in a sensitive Eucalyptus hybrid led to an improved frost tolerance (Navarro *et al.*, 2011).

The present paper is focused on the study of the natural variability of both cold tolerance estimated in a population of French *E. gunnii* families provided by FCBA breeders (Forêt Cellulose Bois-Construction Ameublement) and variation of EguCBF1a sequence. The identification of polymorphisms is increasingly used in woody plants to perform association studies by sequencing candidate genes strongly involved in the response to abiotic stress (Neale & Kremer, 2011).

Materials and Methods

Plant material

E. gunnii genotypes are provided by the FCBA as rooted cuttings in Jiffy plugs. These plants were kept in quarantine before transfer into the growth chamber where they are cultured in standard conditions, with a photoperiod of 16 h at 25°C/8h at 22°C and 80% humidity and an irradiance of 115 µE. Seedlings were supplied from uncontrolled crossings and harvested on different plots of FCBA. A family is composed of full brothers from the same batch of seeds harvested from a mother. Populations provided by FCBA are composed of single copies of each genotype which were first phenotyped and some of them were selected. Lately, FCBA breeders provided us with clones corresponding to each selected genotype which were evaluated again for cold tolerance.

Acclimation program

Plants are subjected to chilling temperatures in a growth chamber (CRYONEXT, Montpellier, France) with a photoperiod of 8 hours day/16h night, a thermoperiod of 25°C day/22°C night and an illumination of 45 µE. The acclimation program is shown in Figure 1. Freezing tolerance was measured by ion leakage method before acclimation (D0), during and at the end of acclimation (D7 and D20/27).

Freezing measurement

Freezing damage was determined as electrolyte leakage after freezing of detached foliar disks. Briefly, 6 foliar disks of 8 mm diameter were cutted from three different leaves and placed in a tube containing 20 mL of distilled water. After one night at the cold room, the inital conductivity (IC) is measured. Tubes were then transferred to a thermoregulated bath cooling at a rate of 2.5°C.h^{-1}. At -1°C, an ice crystal is introduced into the tubes to initiate homogeneously ice formation. At -6°C, tubes are removed and thawed slowly at 4°C overnight. The total conductivity is measured (TC)

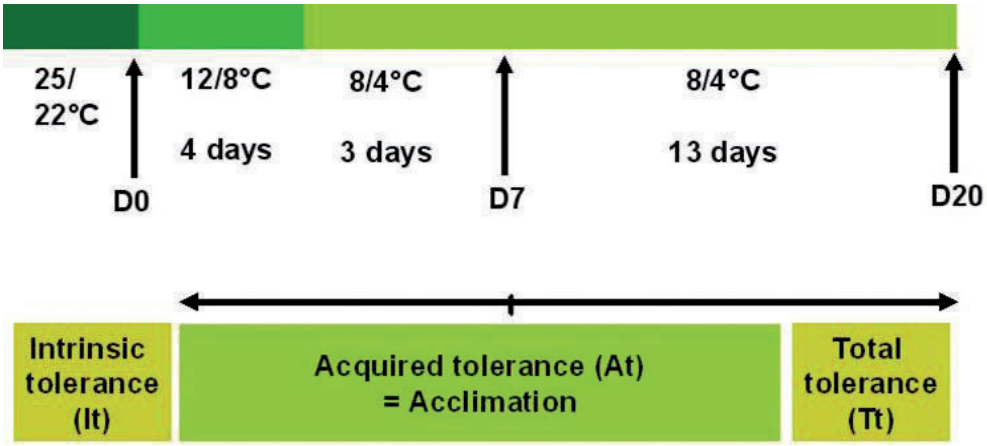

Figure 1: Cold culture program. D27, not shown in this figure, corresponds to the addition of 7 days at 8/4°C. Total tolerance corresponds to the sum of It and At.

and the tubes are placed at -80 ° C for 24 hours to completely destroy the leaf cells. After thawing (24 hours after the release of -80 °C), the final conductivity (FC) is measured. The relative conductivity (RC) is calculated as follows: RC = (TC - IC) / (FC - IC). In *Eucalyptus*, it is considered that a relative conductivity of 0.5 corresponds to 50% of destroyed leaves (Tibbits & Reid, 1987). Thus we can calculate the percentage of cell viability after leaf freezing: (1-RC) x100.

Genotyping of CBF genes

Genomic DNA was extracted from leaves using DNeasy Plant Mini Kit of Qiagen (Hilden, Germany). Primers were designed in the 3'UTR using the PRIMER EXPRESS 2.0 software from Applied Biosystems (Courtaboeuf, France). PCR reactions were performed and then the products were purified. *pGEM-T Easy* vectors were used for *cloning* PCR products, and 10 bacterial transformed colonies were then sequenced with the capillary sequencer ABI 3730 DNA analyzer. Sequence alignment and SNP detection were performed manually using Genalys software (Takahashi *et al.*, 2003). Discrimination of haplotypes is then performed and they are sorted in order of their frequency on total sequences and compared to their frequency within each genotype. The nucleic acid sequences are translated into proteins with "Multiple translation" (http://bioinfo.hku.hk/services/analyseq/cgi-bin/traduc_in.pl). Sequence alignments were performed with CBF "Multalin" at INRA in Toulouse (http://multalin.toulouse.inra.fr / Multalin / multalin.html), providing similarity and identity rates between proteins. Finally, the prediction of cis-elements of the CBF promoters was obtained using the software PLACE (http://www.dna.affrc.go.jp/PLACE) (Higo *et al.*, 1999).

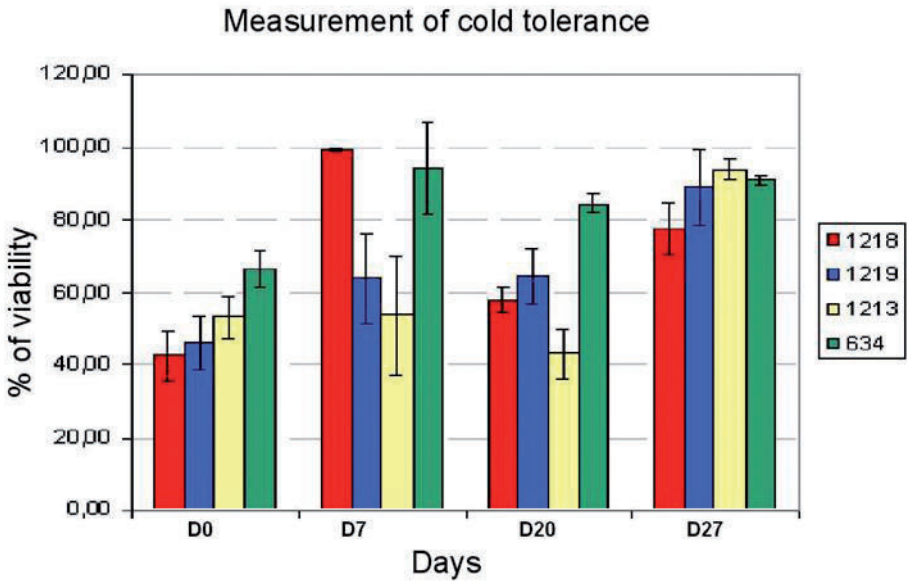

Figure 2 : Percentage of viability of the selected genotypes. Values are the mean of 3 independent plants per genotypes. Error bars represent standard errors.

Results

Freezing tolerance phenotype

In *E. gunnii*, our results show a high variability both in intrinsic and cold acclimation between the tested genotypes. In agreement with the litterature, the intrinsic tolerance and acclimation capacity proved independent since some of the best genotypes for intrinsic tolerance were found to acclimate very well, others poorly and vice versa (Stone *et al.*, 1993). Evaluating all these parameters (intrinsic tolerance D0, acclimation capacity D20 and speed of acclimation D7) allowed us to classify the studied genotypes. The aim was to find the ideal profile for the breeders. Among the 34 studied genotypes, we selected 4 of them differing in their intrinsic tolerance and extent of acclimation. Since some genotypes continue the acquisition of tolerance, we designed a new program for acclimation in which the acclimation phase was extended for 7 days.

Using these new conditions, a new phenotyping was conducted for the four selected genotypes available as rooted-cuttings (Figure 2). Although the obtained values vary between the two measurements which is probably due to the physiological state of seedlings, the tolerance profile remains the same. These clones with interesting features were selected to perform further gene expression studies.

CBF gene polymorphisms

By using primers amplifying the EguCBF1a, six different sequences were identi-

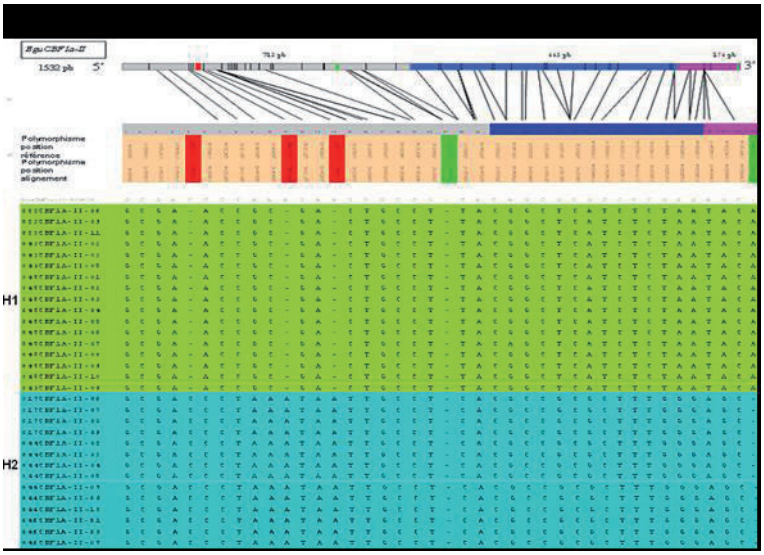

Figure 3 : Haplotypes identified after SNP alignement and classified according to their frequency.

fied namely CBF1aI to VI (Marque, 2008). Due to the high homology between these sequences, it was difficult to design six pairs of specific primers amplifiying each form. Thus, a single pair has been designed and considered specific for EguCBF1aII. The induction of this gene after a cold shock was verified by expression analysis (data not shown). On the total of the genotypes already phenotyped for their cold tolerance, a search of DNA polymorphism was conducted with the aim of identifying haplotypes. SNP were detected on both the codant and the promoter regions of the EguCBF1aII. Polymorphisms were only considered when present on at least 10% of the aligned sequences. This search have revealed 36 SNPs and 5 indels (insertions 2 and 3 deletions). By aligning the polymorphisms (36 SNPs and 5 INDEL) 98 haplotypes were obtained. Only 18 of them were selected for further work based on their frequency (Figure 3).

Promoter and protein polymorphisms

The most remarkable polymorphisms in the promoter region correspond to a deletion of 10 base pairs (bp) and an insertion of 8 bp. In addition to these two major changes in the promoter region, 19 SNPs/2 INDELs were also found, while only 14 SNPs are detected in the coding region, and 3 SNPs/1 INDEL in the 3'UTR.

The prediction of the cis elements in the promoter region of the 18 chosen haplotypes was conducted using the PLACE software (Higo *et al.*, 1999). Among the well known cis elements involved in cold tolerance, MYC, RAV1, MYB, ABRE and CRT/DRE/LTRE were studied. Their number varies with that of INDELs and SNPs in the sequence of each haplotype. Among the 18 haplotypes, eight differ by the predicted elements in the promoter sequences. Genotypes belonging to these eight haplotypes appear to have a characteristic cold acclimation profile. Interestingly, the prediction

of the CRT/DRE/LTRE cis element in the promoter region of the CBF gene may suggest an auto-regulation.

The comparison of amino acid sequences of the coding region of the 18 haplotypes confirms the conservation of amino acids in the AP2 domain and the COOH-terminal domain. It also shows the modification in some residues resulting from polymorphisms. Two haplotypes, with two residues changes in their C-terminal part, are interesting because they include all the sequences of two genotypes that share a low intrinsic tolerance.

Discussion

This study allowed dissecting cold acclimation process in *Eucalyptus* and identifying the most discriminating parameters. Among them acclimation speed looks very variable and important for the survival in the field. Genotypes significantly differing for their tolerance could be chosen. In parallel, the identification of SNP molecular markers likely to have an impact on cold induction could be detected on promoters of some of these genotypes. Studies have evidenced a correlation between gene expression of CBF1 and 2 of *A. thaliana* and freezing tolerance in different accessions after 14 days of acclimatization (Hannah *et al.*, 2006). A recent study in *Setaria italica* has associated a nucleic mutation (G instead of A) in the SiDREB2 gene to drought tolerance (Lata *et al.*, 2011).

This preliminary study represents a significant step towards the identification of markers for selection of cold tolerance in *Eucalyptus*. Future studies will focus on the relationship between changes in cis elements of the promoters and expression of CBF as well as the cold tolerance of studied genotypes.

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Molecular Mapping of Fusarium Resistance QTL in the Japanese Wheat Cultivar Nobeoka Bozu

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Abstract

The molecular background of the FHB (Fusarium head blight) resistant Japanese Nobeoka Bozu landrace was mapped in the Ringo Star//Mini Manó/Nobeoka Bozu/3/ Avle (n=163) DH population. Phenotyping was made in Hungary and Norway applying spraying method. Genotyping was performed in Fargo (USDA-ARS) with 96 SSR markers. The results show that the identified FHB and FDK (Fusarium damaged kernel) resistance QTL (Quantitative trait locus) region on the 3BS (*Xbarc75-Xcfd79*) should include more than one medium effective QTL in the Nobeoka Bozu variety; a presence of a QTL cluster is assumed. At the centromere of the 3B (*Xwmc808-Xgwm77*), an additional QTL was identified. The QTL on the 2B (*Xgwm388-Xbarc101*) was linked to both plant height and *Fusarium* resistance. Correlation analysis showed significant association between the two traits which assumes morphological resistance. The *Fusarium* resistance QTL on chromosome 5A (*Xgwm129-Xbarc180*) was also validated. On the 6B chromosome awnedness (effect of B2 gene) and plant height showed significant association with the region flanked by the markers *Xgwm88* and *Xgwm219*. The resistance of Nobeoka Bozu and the Chinese landraces resistance sources show differences on the chromosomes 3B and 6B.

Keywords: *Triticum aestivum*, Fusarium Head Blight, Fusarium damaged kernel, resistance, QTL

1. Introduction

Fusarium head blight (FHB) can cause serious losses in yield quality and quantity

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of small grain cereals. Severe epidemics occur in almost every 2-4 years not only in Europe, but also in the USA, Canada or China. In Central Europe *Fusarium graminearum* the most frequent pathogen, but *F. culmorum* can also be isolated from wheat. Although up to 17 species have been associated with the disease [1]. Most of the wheat varieties grown in the EU are susceptible to this disease. Crop management and chemical control to prevent the disease and associated mycotoxin contamination are either not available or not feasible. The development of resistant varieties is the most reliable and environmentally proper to combat this disease [2]. Using resistant wheat sources from Asia (i.e.: Sumai 3) in the breeding process is a sound way to reach this goal and introgress resistance genes into new varieties. Investigating wild types of wheat is an emerging research area to find highly effective FHB resistance genes leading almost to an immune phenotype [3, 4].

The Japanese Nobeoka Bozu is one of the Asian resistance sources, which is often iniquitously unheeded from breeding and *Fusarium* resistance experiments. The Nobeoka Bozu is an agronomically poor, ill-adapted landrace and the straw is thin, the heads and grains are small (thousand kernel weight is about 25-27g), although it has comparable FHB resistance to Sumai 3 [5, 6]. Among the breeding lines derived from the crosses of Nobeoka Bozu, several highly resistant lines were identified, which had special value for winter wheat programs [5].

Some morphological traits like plant height, spikelet density within the head, awnedness can influence *Fusarium* infection, as passive resistance mechanisms [7]. It is not yet clear that the *B2* awnedness gene (chromosome 6B) of the awnless Nobeoka Bozu is linked to *Fusarium* resistance or not [8, 9]. The association was not clear either in the case of the *B1* gene (chromosome 5A), investigated in the Sumai 3 [8, 10].

It is well know that the Nobeoka Bozu has highly effective QTL on the 3BS chromosome, though it has different allele sizes compared to Sumai 3, nonetheless it has good Type II resistance [11]. Bai et al. [12] also stated that the Japanese and Chinese resistance sources have different banding pattern with the markers in the major 3BS QTL region.

In the Nobeoka Bozu from 3 resistance QTL – including the 3BS and 6B QTL region – two were unique and one was identical with the Sumai 3 [9, 13]. Some studies did not find differences between the fragment pattern of Nobeoka Bozu and Sumai 3 on the 3BS and 6B [14, 15]. However other genetic diversity studies showed that they have no common SSR allele on either 3BS QTL [12, 16, 17] or 6BS QTL [17].

As there are still many questions about the molecular background of the Nobeoka Bozu *Fusarium* resistance mechanisms, it is highly important to find answers, identify and clarify the resistance QTL of this Japanese variety. In this study emphasis was placed on genotyping the Noboka Bozu *Fusarium* resistance QTL on chromosomes 2B, 2D, 3BS, 3Bc, 5A and 6B in a Ringo Star//Mini Manó/Nobeoka Bozu/3/Avle DH population.

2. Materials and Methods

2.1 Plant Materials

The Ringo Star//Mini Manó/Nobeoka Bozu/3/Avle F2-derived doubled haploid (DH) population of 163 lines was developed from the cross between the line Ringo Star//Mini Manó/Nobeoka Bozu and ‘Avle’ using the wheat × maize system [18]. The

FHB resistant cross of 'Ringo Star'//'Mini Manó'/'Nobeoka Bozu' was generated by the Cereal Research Institute, Szeged, Hungary. 'Avle' is a susceptible spring wheat cultivar with the pedigree TW232-62/'Kadett'//'Nemares' from the Swedish breeding company Lantmännen SW Seed Ltd.

2.2 Field experiments and Fusarium resistance evaluation in Norway

The 132 spring types of the DH population was planted at Vollebekk Research Farm in Ås (Norway) in one season (2004). Seed were sown in May in hill plots, 40 by 45 cm apart in three replicates following a randomized complete block design. A bundle of about 10 to 15 heads per plot were inoculated with hand sprayers at full flowering by spraying 10 to 15 ml of a conidial suspension at 1×10^5 spores ml^{-1} of F. culmorum. The inoculum consisted of a mixture of five isolates and was produced as described by Semagn et al. [19]. Inoculated heads were covered with a transparent polyethylene bag as described by Mesterhazy [7] for 2 to 3 days (45 day degrees [d°C]). The proportion of infected spikelets per bundle was estimated visually using a linear scale from 0 to 100%. Fusarium head blight severity was scored three times on the basis of constant temperature sums after inoculation (d°C). The mean FHB severity of the three scores was used for further analysis. Plant height and awnedness was also scored.

2.3 Field experiments and Fusarium resistance evaluation in Hungary

The 163 lines of the DH population were evaluated in the nursery of Cereal Research Non-Profit Ltd. in Szeged (Hungary) over two seasons (2008 and 2011) using individual *F. graminearum* and *F. culmorum* isolates. Seed was sown in all experiments in mid October, using Wintersteiger Plot Spider planter (Wintersteiger GmbH, Ried, Austria), at the usual sowing time of winter wheat. The plots (i.e. genotypes) were planted in two replications in a randomized complete block design. No winter damage was observed in these experiments. Each replicate consisted of a 1.5 m long row. Each genotype was inoculated individually with two to three isolates of either *F. graminearum* or *F. culmorum*. In the experiments altogether five isolates were used, however one isolate of 2011 was not pathogenic enough, so it was omitted from further analysis. Inoculum production and inoculation procedure as well as the observations of FHB disease severity and FDK were done according to Szabó-Hevér et al. [20].

In each year, plant height was measured as the distance from the soil surface to the top of heads excluding awns. The date of heading and anthesis were also recorded as the number of days from 1 January to heading or anthesis. In addition awnedness was also recorded.

2.4 Genomic DNA extraction

DNA was isolated from seedling leaf tissue Wizard® Genomic DNA Purification Kit (Promega Corporation, USA) according to manufacturer instruction. The quality and quantity of DNA was measured with NanoDrop 1000 Spectrophotometer (Thermo Scientific Company).

2.5 Molecular markers

For molecular mapping 96 SSR markers were selected according to the map of Sommers et al. (2004). PCR was performed with 10 µl per reaction containing 1X PCR buffer (New England Biolabs, Inc. Beverly, MA), 0.125 mM dNTPs, 0.4 pmol forward primer, 0.3 pmol reverse primer, 3.0 pmol of M13 primer labeled with one of the four fluorescent dyes (6-FAM, VIC, NED, and PET), 0.05 units/µl Taq DNA polymerase (NEB), and ~75 ng genomic DNA. The PCR reaction mixture was initially denatured at 94°C for 10 min, followed by 40 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, with a final extension step of 72°C for 5 min and 4°C indefinitely. The PCR thermal cycling was performed using the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The PCR products amplified with four different dyes (6-FAM, VIC, NED, and PET) were multiplexed to a final volume of 10 µl, including 0.14 µl GeneScan-500 LIZ® size standard (Applied Biosystems) and 6.86 µl Hi-Di™ Formamide (Applied Biosystems). Mixed PCR products were denatured at 94°C for 5 min and chilled on ice. PCR products were separated by capillary electrophoresis using the ABI 3130xl Genetic Analyzer (Applied Biosystems). Separated SSR-amplified fragments were analyzed using GeneMapper software v3.7 (Applied Biosystems) as described in the user manuals.

2.6 Statistical analysis

Statistical analyses were made by SPSS 15.0 software "Descriptive statistics" function to calculate means, minimum and maximum values, percentiles, standard deviation, to test the normal distribution of the FHB severity and FDK data. The FHB severity and FDK data for each fungal isolate from different years were analyzed as single experiments (epidemic situations), because there are no races within *F. graminearum* and host resistance is non-specific for different *Fusarium* spp. [7, 21]. Therefore the epidemics caused by either *F. graminearum* or *F. culmorum* could be analyzed together.

2.7 QTL analysis

Linkage groups were constructed using JoinMap® 3.0 [22] and interval mapping was done using MapQTL® 5 [23]. Linkage groups were established by using a maximum recombination fraction of 0.45. The permutation tests (determined by 1000 iterations) indicated 1.9 as minimum LOD score on a P=5% significance level. Interval mapping (IM) was made with the phenotypic data of single experiments, with the mean values of *F. graminearum* and *F. culmorum* inoculations and overall means for FHB severity and FDK rates of all the selected isolates, as well as with heading date, plant height and awnedness.

3. Results

3.1 Field data

All the field data used for mapping showed normal distribution in all experimental environments and epidemic situation, only one isolate of 2011 showed low pathogenicity, so it was discarded from further analysis. The parents showed the expected resistance level in the population. The FHB mean overall the epidemic situations was

	FHB	FHB1	FHB2	FHB3	Date of Inoculation	Plant height
FHB1	0,891**					
FHB2	0,875**	0,679**				
FHB3	0,881**	0,669**	0,616**			
Date of inoculation	n.s.	n.s.	n.s.	n.s.		
Plant height	n.s.	n.s.	n.s.	n.s.	0,332**	
Awnedness	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table 1. Correlations between FHB (Fusarium head blight), date of inoculation, plant height and awnedness. Norwegian mean data. (FHB1, FHB2 and FHB3 are replication within one environment)

	FHB	FDK	Date of Inoculation	Plant height
FDK	0,768**			
Date of inoculation	n.s.	n.s.		
Plant height	-0,306**	-0,217**	n.s.	
Awnedness	n.s.	n.s.	n.s.	n.s.

Table 2. Correlations between FHB (Fusarium head blight), FDK (Fusarium damaged kernel), date of inoculation, plant height and awnedness. Hungarian mean data.

11.2% (population range: 0.9 - 38.2%) in Hungary and 14.5% (population range: 3.7 – 40.3%) in Norway. The FDK mean overall the epidemic situations was 38.1% (population range: 2.5 – 88.8%) in Hungary. For plant height the population range in Norway was from 54 cm to 98 cm, and in Hungary from 85 cm to 145 cm.

The accuracy of the experiments is clearly shown in the correlation between the traits. The correlation between the FHB severity and FDK rates was significant on the P=1% level (Table 1). However some significant correlations were observed between the *Fusarium* symptoms and plant height in the case of the Hungarian data (Table 2).

3.2 Linkage analysis

From 96 markers 39 were polymorphic for the Nobeoka Bozu allele. The linkage analysis revealed two groups on chromosome 3B including one 22.2cM long region on the 3BS (*Xbarc75-Xcfd79*) and another 41.9cM long region on the 3Bc (*Xwmc808-Xgwm77*). On chromosome 5A (*Xgwm129-Xbarc156*) a 25.1cM long region was identified. Additional three linkage groups were detected on chromosomes 2B (*Xgwm388-Xbarc101*), 2D (*Xbarc228-Xgwm539*) and 6B (*Xgwm88-Xgwm219*).

3.3 QTL mapping

The most prominent LOD curve in the 3BS (*Xbarc75-Xcfd79*) region was obtained

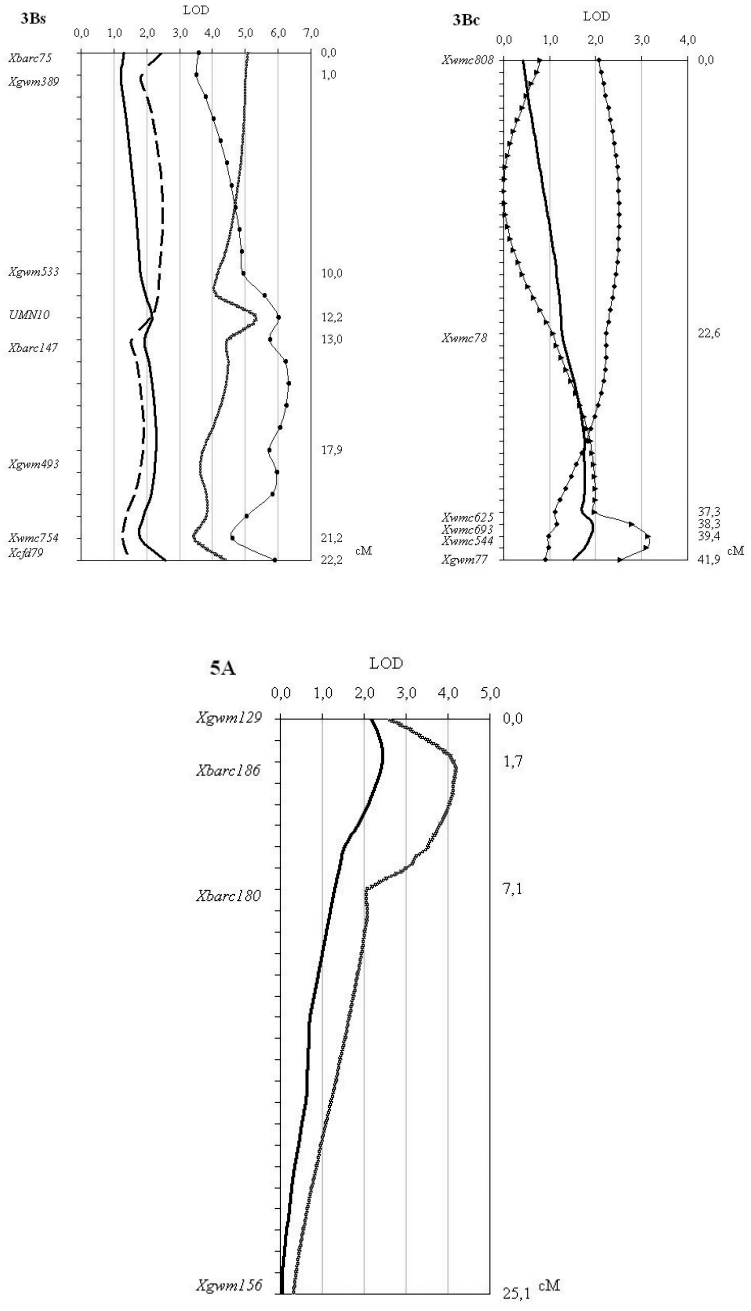

Fig. 1. Genetic map of QTL associated with FHB (Fusarium head blight), FDK (Fusarium damaged kernel), plant height and flowering date.

with FDK data and the Norwegian FHB data. LOD values were found close to the significant level with the Hungarian FHB and plant height data (Figure 1). Markers on the 3Bc (*Xwmc808-Xgwm77*) associated with FDK and flowering date. A QTL region on the 5A (*Xgwm129-Xbarc180*) linked to FHB resistance was verified across the experiments of the two countries. This marker region was also associated with FDK in the case of one inoculum, but this was not identifiable with mean FDK data. Other QTL with less markers were identified associating with plant height on chromosomes 2B (*Xgwm388-Xbarc101*) and 6B (*Xgwm88-Xgwm219*). These QTL were able to be validated with the records of both countries. QTL on chromosome 2D (*Xbarc228-Xgwm539*) (not validated) and 6B (*Xgwm88-Xgwm219*) (validated) were linked to awnedness.

4. Discussion

In the Ringo Star//Mini Manó/Nobeoka Bozu/3/Avle population Fusarium resistance QTL were identified on chromosomes 3BS, 3Bc and 5A of the Nobeoka Bozu. On 3BS the well characterized, Sumai 3 (Chinese) derived *fhb1* locus was identified also in our Nobeoka Bozu (Japanese) parent. This result is in line with those that say the 3BS QTL is identical between the two resistance sources [15]. On the other hand more LOD peaks were identified, other than the one at the *fhb1* locus. This let us to presume that in this region not only one, but more FHB and FDK resistance QTL are present, which would explain the inconsistent and unclear conclusions about the Nobeoka Bozu 3BS QTL in the *Fusarium* literature [5, 9, 11, 12, 13, 24]. The significant LOD level in this chromosome region with plant height data must be explained with the correlation between plant height and FHB and FDK. Therefore it is not supposed that there is a QTL influencing plant height at this chromosome section.

FDK resistance QTL on the 3Bc (*Xwmc808-Xwmc625*) was also identified. In the *Xwmc625-Xgwm77* chromosome region the markers showed association with flowering date and a low association with FHB resistance. This QTL is rarely detected in the mapping projects, although McCartney et al. [17] got similar results with the Japanese resistance source Nyu bai, which variety is genetically very close to Nobeoka Bozu [11, 12].

The FHB QTL on chromosome 5A was validated with the data of both countries (Norway and Hungary). This QTL is also present in many other resistance sources like Sumai 3 (Chinese), Nyu bai (Japanese), Frontana (Brazilian), Ernie (European) [4]. Ban [25] reported that one resistance gene in Sumai 3 is linked to the *B1* locus on chromosome 5A, coding suppression of awns. In our study the FHB resistance QTL on 5A was not linked to awnedness. These results are in line with those of Buerstmayr et al. [10], who found FHB resistance QTL on 5A in the Sumai 3 derived CM82036 and it was not associating with the *B1* awnesdess gene.

The *B2* gene on chromosome 6B coding awnedness was also published as a locus, which might have effect on FHB resistance [8, 9]. Ban and Inagaki [9] investigated a DH population from the F1 cross of Nobeokabozu (Nobeokabouzu-komugi) and Sumai 3. Following a chi-square test they stated that Nobeokabozu harbours three dominant genes for the resistance, of which two are unique and another gene is identical with the one of Sumai 3.They also stated that awnedness was controlled by one suppressor gene *B2* of Nobeokabozu, which caused to cancel out the effect of a

resistance gene either Nobeokabozu or Sumai 3. In our study association between the *Xgwm88-Xgwm219* marker region on 6B and awnedness was found. This chromosome segment did not flank to any FHB or FDK resistance QTL, which is in line with the results of Ban and Inagaki [9].

Our results indicate that Nobeoka Bozu harbours different QTL from the Chinese resistance sources (i.e. Sumai 3) on chromosome 3Bs (QTL cluster) and 6B.

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Quantification of Allele Dosage in Tetraploid Roses

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Summary

Many important crops (wheat, potato, strawberry, rose, etc.) are polyploid. This complicates genetic analyses, as the same locus can be present on multiple homologous or homoeologous chromosomes. SSR markers are suitable for mapping in segregating populations of polyploids as they are multi-allelic, making it possible to detect different marker alleles of the same locus on multiple homologous r homoeologous chromosomes. If a SSR primer pair gives fewer alleles than the ploidy level, quantification of allele dosages increases information content. We show the power of this approach for the generation of a genetic map in a auto-tetraploid garden rose population. Alleles were scored quantitatively using the area under the peaks in ABI electropherograms, and allele dosages were inferred based on the ratios between the peak areas for two alleles in which these two alleles occurred together. We hereby start with some reference cases in which the ratio's are determined between alleles that are in simplex condition. We thus resolved the full progeny genotypes, generated more data and mapped markers more accurately, including "null" alleles. The maps will be used for locating QTLs for winterhardiness in tetraploid roses.

Introduction

The frequent occurrence and widespread distribution of polyploids suggest that they play an important role in evolution. Roughly 50% of angiosperms and 44-95% of ferns and fern allies have a polyploid origin (Luo et al., 2006). Polyploidization has played a major role in a plant evolution by increasing gene redundancy and morphological complexity. As a result, polypoids are often more adaptable and show increased tolerance to environmental conditions (Xie and Schizhong, 1999; Gar et al., 2011). Basically there are two classes of polyploids: autopolyploids and allopolyploids. Allopolyploids or bivalent polyploids originated from at least two different species. Preferential pairing of homoeologous chromosomes during meiosis leads to the di-

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somic inheritance. Multivalent polyploids or autopolyploids may be derived from a single ancestral species, mostly through duplication of the genome. In autopolyploids chromosomes pair randomly in meiosis, leading to polysomic inheritance. Sometimes even more than two homologous chromosomes pair with each other, forming tetravalents, which may lead to 'double reduction'. In reality, the type of pairing may also vary among chromosomes, which makes genetic analysis more complex (Li et al., 2010, Stift et al, 2008).

Garden and cut roses are tetraploids with small chromosome number and genome size. Despite roses being the most important ornamental, and the huge development of genomics, little is known of rose genetics, largely due to varying ploidy level among species, high degree of heterozygosity and specific sexual reproduction. Rose genetics influences breeding success, especially if we keep in mind low seed germination rate (Gudin, 2000; Yan et al, 2005).

In the era of genomics marker-assisted breeding is rapidly becoming an important tool as it may improve the choice of parents to cross with and also significantly shorten the breeding cycle. In major diploid crops molecular markers are routinely used for genetic map development and mapping of quantitative and qualitative characteristics. In sharp contrast, the application of molecular tools in breeding of autopolyploid species is still limited. This is largely due to the complexities of gene segregation and recombination during meiosis, namely: multiple allele segregation, variable allele dose, double reduction and mixed bivalent and quadrivalent pairing among homologous chromosomes (Luo et al., 2006). According to Luo et al. (2001) estimates of recombination frequencies based on multi-allelic markers are up to four times as informative as the best estimates from dominant markers. Thanks to their multi-allelic nature SSRs have been widely used for plant genome analysis (Song et al, 2011). Unfortunately, many of the advantages of codominance are lost in the study of polyploids if there are fewer alleles and if the allele dosage would not be determined (Pfeiffer et al., 2011).

Esselink et al. (2004) proposed the MAC-PR procedure for dose assessment in autopolyploids. In some cases, due to differences in amplification among alleles, quantitative scoring is not possible for some marker loci. Here we extend this procedure, that allows us to cope with the dose effect, even in case of differences in amplification efficiency among alleles of a marker locus. This makes it possible to fully assign the allele configuration of any SSR marker locus in a more accurate way, including assignment of null alleles.

Material and methods

The material consisted of three segregtating mapping populations derived from crosses between Morden Centennial, Nipper, Red New Dawn and Winchester Cathedral. Morden Centennial is from a Canadian breeding program for winterhardy garden roses, and was crossed with the three European varieties in order to introgress winterhardiness. The smallest population consists of 42 seedlings.

A set of 23 SSR markers was selected from literature (Debener et al., 2001; Esselink et al., 2004;; Koning-Boucoiran et al., 2012). SSR were amplified by multiplex- or single PCR according to Esselink et al, 2004. The NED-, HEX- or 6-FAM- labelled products were detected using an ABI Prism 3700 DNA analyser (Perkin Elmer Biosy-

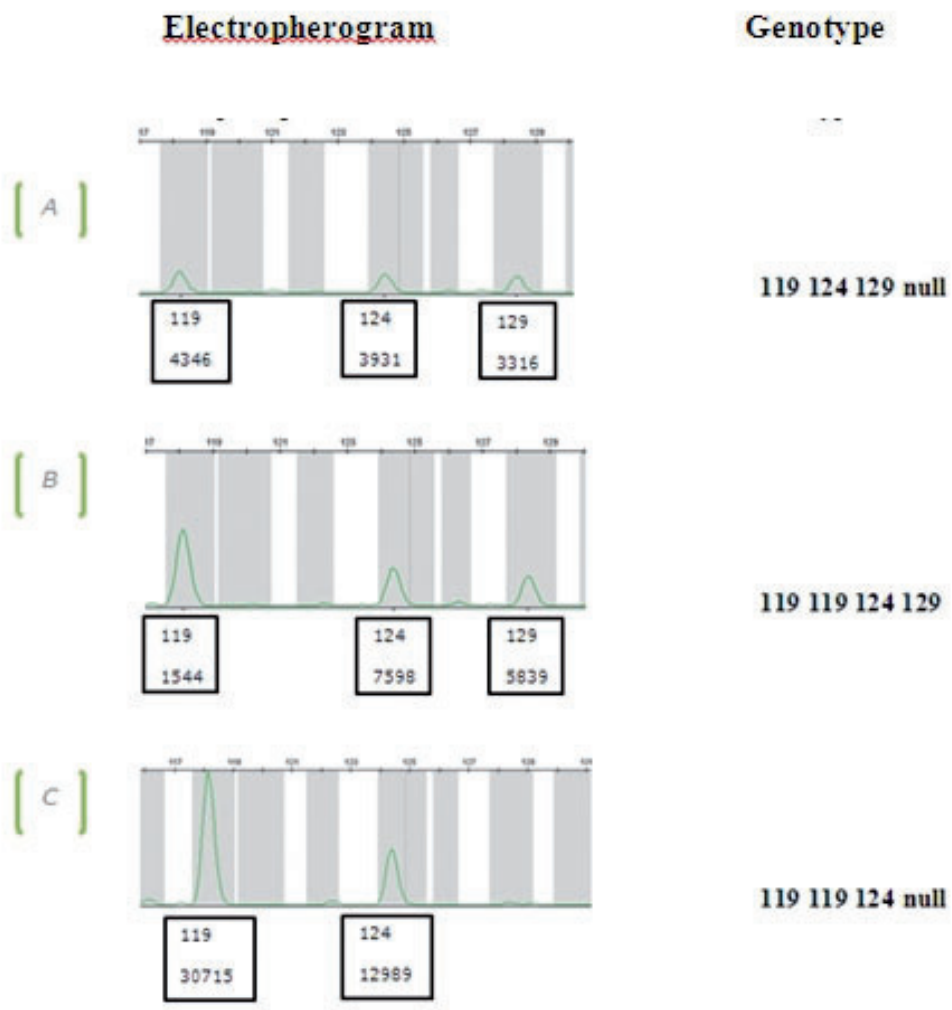

Figure 1. ABI electropherograms for marker RhB303 tested on three progenies of Morden Centennial x Red New Dawn. The values in the boxes indicate the size and area of the related allele (first and last line respectively).

stems, Foster City, California). Fragment sizes and peak areas were automatically determined using GeneMapper.

Results

Procedure. The ABI platform generates electropherograms in which each allele is shown as a peak. As garden roses are tetraploids the number of expected peaks varies from one to four. Observed electropherograms show a trend of slightly decreasing peak

height with increasing allele size. On top of that alleles can have different success of amplification. Alleles amplified using 2nt-SSRs often have one or few stutter bands. After selecting all real alleles and discarding stutter bands (bands that proceed the real allele by steps of 2-3 nt and that have much smaller areas as the real allele), the areas under the peaks are exported to an Excel table.

The first step in quantification of allele dosage is determination of amplification ratios between alleles. Next, the ratios for single dosages are determined, as these serve as reference in the interpretation of all other cases. Hereto data are filtered in Excel for all cases in which an offspring plant has four alleles. Usually an allele pair is represented by several samples. Their ratio's usually fall within a limited range.. On the base on these single dose amplification ratios, expected ratios for other allele dose combinations are calculated.

Secondly, for each pair of alleles the distribution of their ratio's across the entire progeny was examined for separate categories. In perfect case amplification ratios group around values for single dose or as double or triple multiplication of these values.

Thirdly, a dose is assigned to each allele of a progeny, and the full genotype is established, Next, the consistency of these proposed genotypes are checked for their consistency to the allowed maximum number of alleles, which is four for an auto-polyploid, and the genotypes of the parents (Van Dijk et al. 2012).

As an example we show a procedure to score alleles for the SSR marker RhB303 (Fig 1). IAmplification ratios between alleles that are present as simplex are: 1.2 (119/124), 1.3 (119/129), and 1.1 (124/129). Case A has three alleles with amplification ratios 1.1 (119/124), 1.3 (119/129) and 1.2 (124/129), which all match to the references for single-dose ratio's. This progeny thus has three visible alleles that are present at single dose. As a tetraploid should have four alleles, the forth allele must be an null-allele. The full allele configuration thus is 119 124 129 null. In case B there are three alleles with amplification ratios: 2.03 (119/124), 2.6 (119/129), and 1.3 (124/129). Two of these ratio's are around double to that of the references for single dose alleles, indicating that one of these alleles (119) must be at double dose. The genotype this progeny thus is 119 119 124 129. Case C shows only two alleles (119 and 124) with an amplification ratio of 2.4, which is twice that of the reference ratio. The allele configuration must thus be 119 119 124 null.

Complicating issues. Overloading, Bleeding, shifts and stutter bands are drawbacks which make quantitative scoring more complex.

Overloading shows up with if the ABI-platform is loaded with an amount of an amplicon that exceeds the software intensity threshold. As a result, the software cannot correctly determine the amount of this amplicon, due to which the presented area is invalid. When one of markers is overloaded its electropherogram interferes with the electropherograms of other markers. It happens as the fluorescence is not a single wavelength and the filter is not monochromatic, and as the software cannot correct for this anymore. If the origin of such peaks would not be recognised, they would be erroneously assigned as alleles of other SSR-markers.

Another common problem that can lead to wrong interpretation is shifting. Occasionally, all peaks of a sample are shifted a few base pairs to the right or to the left. The reason for this is not clear. In such cases, the automated ABI scores have to be manually adjusted. Stutter bands are the phenomenon that the real allele is accompanied by one or more smaller peaks. Stutter bands are fragments one or several

repeats shorter or longer than the real allele. They are produced during amplification of SSR markers, especially long dinucleotide SSRs. They may hamper scoring and valid conclusions when they co-localize with real alleles. However, they can be accounted for in a statistical way, as their area usually is a quite constant proportion of the real alleles.

Discussion

An understanding of allelic configurations is an essential step of plant genetic studies in polyploids. To early 2000s determination of allele dosage in polyploid species has been mostly unsuccessful. The bands have been scored and interpreted as phenotypic banding patterns and no attempts have been made to assign precise allele dose. The era of quantitative scoring in polyploids started with pioneer work of Esselink et al. in 2004, who succeeded to assign allelic configurations of tetraploid roses in five out of six investigated loci, cumulated in 2012 in the full assessment of allelic configurations in allo-octoploid strawberry (Van Dijk et al., 2012).

Using quantitative scoring in populations made of different parents we were able to confirm null allele detection and to resolve allelic configurations for all individuals. In cases when allele amplification ratios indicated presence of null alleles, parental and progeny allele configurations were checked. When null alleles exist their segregation in progeny follow genetic rules. Additionally, in cases when amplification ratios are between two categories (e.g. between 1:1 and 1:2 or 2:1), the configuration can often be deduced through elimination, using parental genotypes as dose information for the other alleles. Finally, stutter bands may hamper scoring and valid conclusions when they co-localize with real alleles. In previous investigations a lot of markers with stutter bands have been discarded what influenced the final outcome.. Correcting peak area for the value of stutter band improved the quantitative scoring and enabled to score neglected markers from the past.

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Psycho-pathological features of teenagers and preteen with or without delinquent behavior

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Summary

The results obtained for 232 subjects with delinquent and non-delinquent behavior confirm some of the results in the literature regarding the association between psychopathological disorders and the presence of delinquent behaviors (Asscher, 2011; Campbell and al., 2004). The study conducted confirmed this association in subjects selected from the Romanian population, less studied in this regard. By using The Empirically Based Assessment Achenbach System Assessment (ASEBA), we have managed to highlight which types of problems and disorders, emotional, social and thinking, are more evident for delinquent adolescents compared to the control group. Other significant predictors for conduct disorder proved to be: social problems, thinking problems, rule braking and aggressive behavior.

Keywords: delinquency, conduct disorder, personality, psychopathology, prevention.

1. Introduction

The relationship between psychopathology and delinquent behavior aspects has been expanded studied among adults. However, the relevance of mental disorders for the conduct disorders training and development of delinquent behavior still requires extensive investigation for adolescents and preteen (Campbell and al., 2004, p 23).

Development trajectory suggests similarities between boys and girls in the manifestation of antisocial behavior. In the case of adolescents and preteen, they are characterized by the fact that in most cases, antisocial behavior are unstable and are limited to one or two contexts, without seriously affecting the functioning of family, school or the child in society, such as the example, theft "to give greater" (Bonchis, 2004). These types of behaviors are unstable, almost disappear after adolescence (Bonchiş, 2004).

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When these behaviors tend to settle into a stable Patten, we raise the problem of establishing a conduct disorder, which can then lead to the development of delinquent behavior.

In this paper we try to highlight which are the psychopathology aspects that distinguish adolescents with delinquent behavior by adolescents without such behavior.

2. Research methodology

Specific aims. In this study we aimed the following specific objectives:

• Testing ASEBA evaluation system to determine whether is able to differentiate between adolescents and preteens with delinquent behavior and those without delinquent behavior.

• Comparing the intensity of emotional problems, social and thinking problems, in three groups of adolescents and preteens: without delinquent history, with delinquent behavior, and with multiple delinquent relapses.

• Comparing the intensity of psychopathology aspects disorders as measured by ASEBA scales built according to the DSM IV-R criteria to three groups of adolescents and preteens: without delinquent history, with delinquent behavior, and with multiple delinquent relapses.

Variables and design

The design of this thesis is one comparative, quasi-experimental, unifactorial intersubjective. The cited independent variable analyzed in this thesis is the presence of delinquent behavior, with the following arrangements: a1 - without offending behavior, a2 - one delinquent behavior and a3 - with multiple delinquent relapses.

The dependent variables included in the study refer to the results of the ASEBA evaluation system scales.

Participants in the study

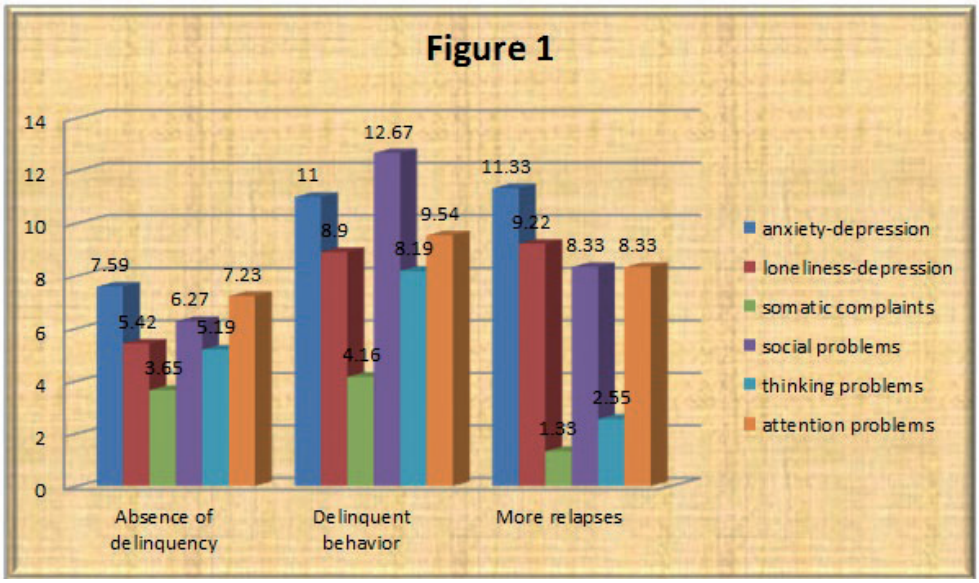
The study involved a total of 232 adolescents and preteens: age from 11 to 18 years (M = 14.26, SD = 1.87). Of these 121 were boys (52.1%) and 111 were girls (47.9%).

Adolescents were contacted and included in the study through three schools in Arad County, both urban and rural, as well by the Placement Center of the Direction of Social Assistance and Child Protection Arad.

The instruments used

The Empirically Based Assessment Achenbach System (ASEBA) by Thomas M. Achenbach and Leslie Rescorla, contains a set of questionnaires assessing competencies, adaptive functioning and problems of children and adolescents (Achenbach et al., 2008, Rescorla, 2005). This was adapted and presenting the Romanian population structure and factorial validity of the adaptations similar to those from other countries (Ivanova et al., 2007).

To measure ASEBA scales fidelity were calculated Pearson correlation coefficients test-retest and coefficients t. For most scales reliability was very high, Pearson correlation indices ranging between 0.80 - 0.90.



Procedure

Clinical interview and questionnaires was conducted after obtaining consent from parents or a representative of Placement Centre of the Direction of Social Assistance and Child Protection Arad, respective subjects aged between 16 and 18 years old. After completing all items of the questionnaire, subjects were asked whether they wish to add other things that were not mentioned during the discussion. Due to lack of literacy or tedious to read / write of many subjects to complete questionnaires was organized form of free discussion, each question being scored properly to response received.

3. Presenting the results

By linking the information about participants and analysis of results from each of the ASEBA evaluation system scales was made a database. The data thus collected and organized were analyzed using SPSS 19.

Figure 1 presents the results obtained in dimensions: anxiety - depression, loneliness - depression, somatic complaints, social problems, thinking problems, attention problems, at the three study groups: group 1 - without delinquent behavior, group 2 - with a single delinquent behavior and group 3 - with multiple delinquent relapses.

Figure 2 presents the results obtained in dimensions: breaking rules, aggressive behavior, internalization, externalization, activities, social, school, to the study group.

Figure 3 presents the results obtained to the dimensions: affective problems, anxiety problems, somatic complaints, ADHD, oppositional behavior, conduct problems according to the delinquency degree.

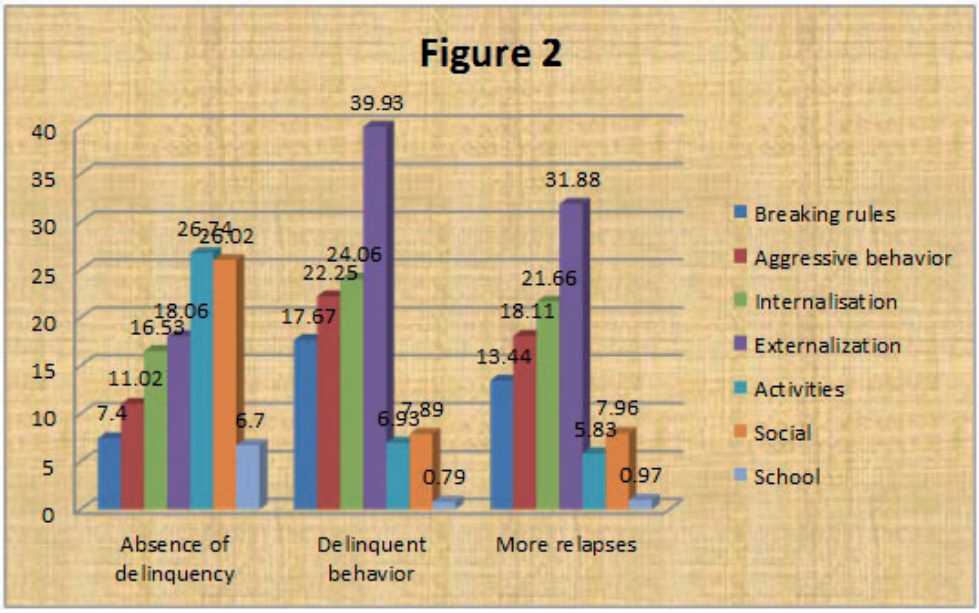


Figure 2
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More relapses
Breaking rules
Aggressive behavior
Internalisation
Externalization
Activities
Social
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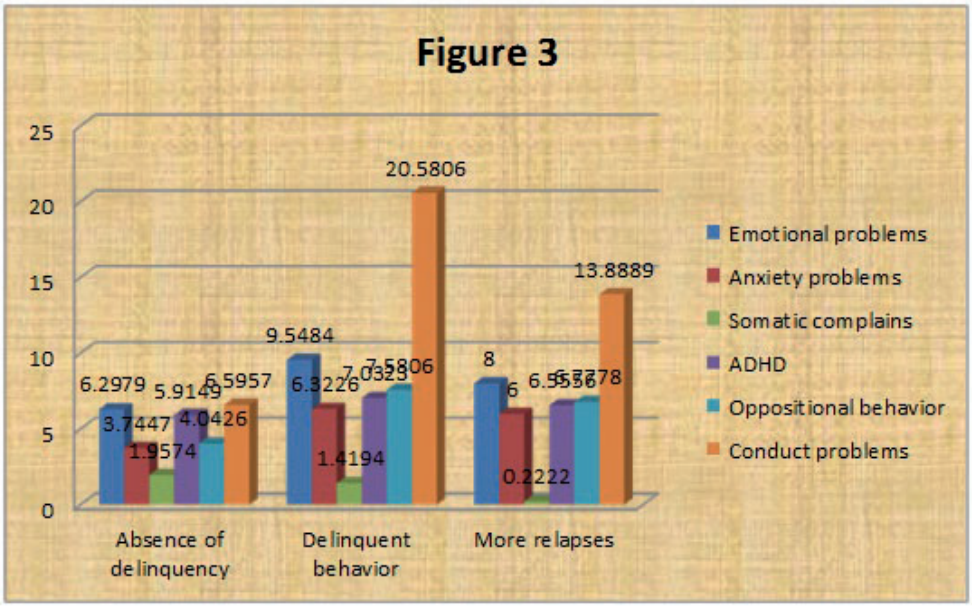
Figure 3
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4.0426
3.7447
1.9574
1.4194
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Absence of delinquency
Delinquent behavior
More relapses
Emotional problems
Anxiety problems
Somatic complains
ADHD
Oppositional behavior
Conduct problems

4. Conclusions

For most dimensions, it supports the hypothesis that participants with delinquent history show a trend of stronger psychopathological disorders than participants in the control group. Among psychopathological aspects, participants in the control group achieved the highest scores for somatic complaints scale conducted by DSM-IV-TR criteria. All adolescents and preteens without delinquent behavior are most involved in extracurricular activities and social, respectively have better school results.

In case of other ASEBA scale evaluation system, the highest scores are obtained by participants with a single delinquent behavior, follow, most often by participants with recurrent delinquent behavior.

These results highlight the trend towards the development of psychopathological disorders for adolescents and preteen with delinquent behavior. However, the degree of delinquency is directly proportional to the intensity of disorder. It suggests two possible explanations:

- The results of participants with lower relapse may be due to dissimulation;
- The lower results of participants with lower recurrence may suggest that for developing recurrent delinquent behavior, adolescents and preteens, even if they present psychopathological disorders or only tend to develop, their intensity should have a lower level.

We could conclude that adolescents and preteens delinquents differ from those without such behaviors by higher levels of development trends of psychopathological manifestations.

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