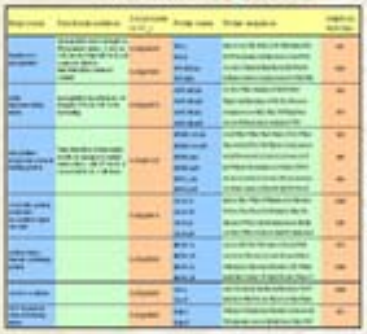
Agarose-Based EcoTILLING as a Tool for Targeted Genotyping in *Oryza* Species

Introduction

EcoTILLING, a derivative of TILLING (Targeting Local Induced Lesions in Genomes), is a reverse genetics tool that is useful in identifying polymorphisms in natural populations at candidate genes of interest. We demonstrate the utility of agarose-based EcoTILLING as a fast and robust technique for detecting SNPs on a mini-core collection of 1536 diverse *O. sativa* and a panel of 95 wild AA genome *Oryza* species accessions. Candidate genes putatively involved in drought tolerance were identified through convergent evidence taking into account functional annotation, altered expression, co-localization with QTLs, and/or allelic shifts under selection. Locus-specific primers were designed using the Nipponbare genomic sequence. Samples were contrasted against two reference lines, Nipponbare (japonica type) and IR64 (indica type). Mismatch patterns detected on agarose were confirmed by sequencing representative samples. The mini-core collection was phenotyped under vegetative drought stress (upland) and control conditions (lowland) in the field. Within isozyme groups there was large phenotypic variation traits related to performance under drought stress, and significant associations established between traits and haplotypes of candidate genes within isozyme groups.

EcoTILLING was confirmed as a useful diagnostic tool for the taxonomic analysis of AA genome wild *Oryza* species. EcoTILLING individual accessions confirmed the taxonomic status of the non-Asian species, *O. barthii, O. longistaminata, O. glumaepatula*, and *O. meridionalis* but not the Asian species *O. rufipogon* and *O. nivara* which were not clearly differentiated from each other.

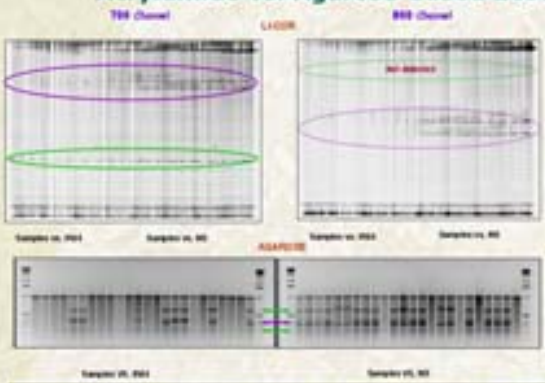
Candidate genes



Plant materials

Extended to 1536 *O. sativa* and 95 wild AA genome *Oryza* species accessions

Acrylamide vs. Agarose-based EcoTILLING



Mismatch detection for the MAPk2 gene on acrylamide (via the LICOR system) and on agarose platforms. Complementary fragments indicating SNPs are highlighted by similarly colored solid and broken lines viewed on the LICOR 700 and 800 channels, respectively. On agarose, corresponding bands are indicated by similarly colored arrows. Samples on agarose correspond to the last 24 samples on the LICOR image.

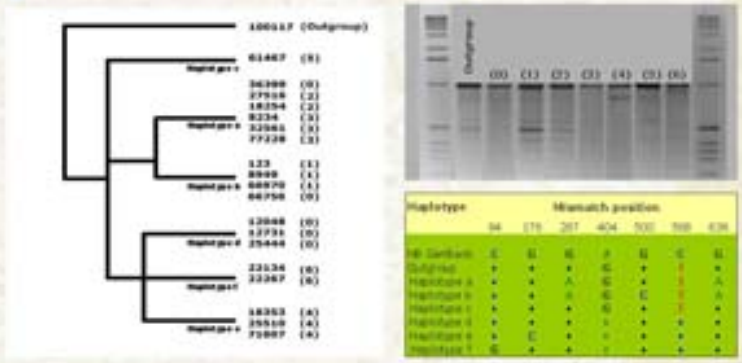
Component/Step	Acrylamide (LICOR)	Agarose
DNA pooling	2 per pool	2 per pool
PCR primer	Labeled (IRD 700 and IRD 800) + unlabeled	Unlabeled
PCR product	100-200 ng	400-500 ng
CEL 1 components		
Celery juice extract (CJE) enzyme	0.02 µl	0.20 µl
CJE buffer	3.00 µl	1.50µl
SUP water	16.98 µl	8.30µl
Digestion	Add 20 µl to PCR product; digest at 45 °C for 15 mins	Add 10 µl to PCR product; digest at 45 °C for 20-25 mins
Post digestion steps	Stop with 5 µl 0.225M EDTA; mix; concentrate; load 0.3 µl sample	Stop with 5 µl 0.225M EDTA; mix; load 8 µl sample
Electrophoresis	Run on LiCOR for 3 to 4 hours	Run on 1.4% agarose for 1.5 to 2 hrs

Results

Pattern detection on agarose and confirmatory sequencing

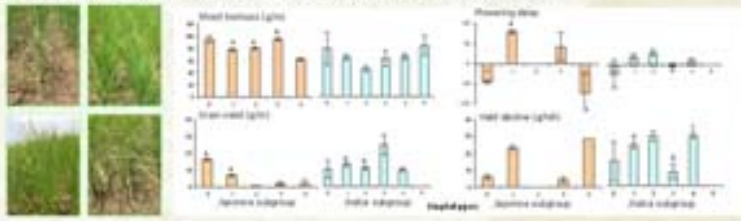
Primer	No. of patterns		Number of accessions				Sampled for sequencing
			With mismatch		Without mismatch		
	vs. IR 64	vs. NB	vs. IR 64	vs. NB	vs. IR 64	vs. NB	
ADF3a	6	3	940	940	895	585	37
ADF3b	6	5	718	730	807	792	28
DREB2	7	10	415	1266	1108	202	40
ERF1	8	6	993	983	541	552	82
ERF3 up	–	–	–	–	–	–	50
MAPK2	5	6	627	1470	909	65	30
TPP	8	8		53		1403	33
BZIP	4	6	1060	840	472	472	19
NAC	8	5	959	1345	636	165	46

Left, mismatch variation patterns for 1536 *O.sativa* accessions contrasted against IR 64 and Nipponbare (NB) detected by agarose-based EcOTILLING. Below, although adjacent SNPs (e.g., mismatch positions 404 and 500 in haplotype b) are not resolved on agarose (pattern 1) haplotypes generated from MR consense tree using Phylip v3.67 for the BZIP gene corroborate with patterns detected on agarose (values in parentheses).



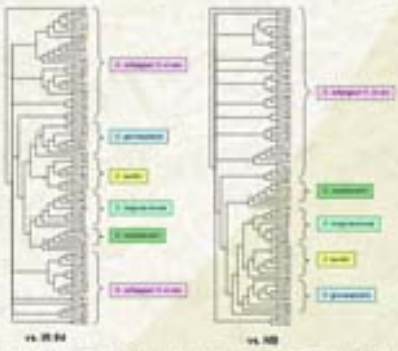
Association genetics

Field screening of the mini-core collection was conducted over three consecutive dry seasons (2003-2006) under vegetative drought stress (upland) and well-watered conditions (lowland).

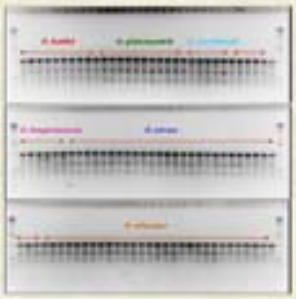


Association between sucrose synthase haplotypes and phenotypes under drought stress in the *Japonica* and *Indica* subpopulations (1-way ANOVA, data for each subpopulation were analysed separately). Bars with different letters significantly differ at the p<0.05 level. Error bars represent ±1 s.e.

Definition of biosystematic relationships in AA genome *Oryza* species



Neighbor joining phylogenetic trees generated through Nei's Distance Method (PowerMarker v3.25) based on mismatch patterns for 7 primers. Numbers on nodes correspond to bootstrap values. Species delineation among the non-Asian species supports current taxonomic classification.



Mismatch patterns of AA genome *Oryza* species accessions contrasted against Nipponbare for the MAPk2 gene. Species-specific mismatches are shown by *O. barthii, O. glumaepatula, O. meridionalis*, and *O. longistaminata*. Overlapping patterns are shown by *O. nivara* and *O. rufipogon*.

Conclusion and future prospects

Agarose-based EcoTILLING is an efficient genotyping tool for the *Oryza* germplasm. SNPs or indels detected on agarose are largely supported by sequence data and may prospectively be useful with associated phenotyping. Its utility as a diagnostic tool in *Oryza* classification can be further explored using primers for other low-copy nuclear genes with broader genome coverage.

M.E.B. Naredo[1], M.B. Gamalinda[1], G.A. Atienza[2], R.J.A. Melgar[1], M.D. Sanciangco[1], C. Raghavan[2], J. Cairns[3], R. Lafitte[1], G. Atlin[2], A. Kumar[2], R. Serraj[3], and K. L. McNally[1]
T.T. Chang Genetic Resources Center[1], Plant Breeding, Genetics, and Biotechnology Division[2], and Crop and Environmental Sciences Division[3], International Rice Research Institute, DAPO 7777, Metro Manila 1301, the Philippines.

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