EcoTILLING: A TECHNIQUE FOR DETECTING NATURAL VARIATION AND A DIAGNOSTIC TOOL FOR BIOSYSTEMATIC STUDIES IN ORYZA

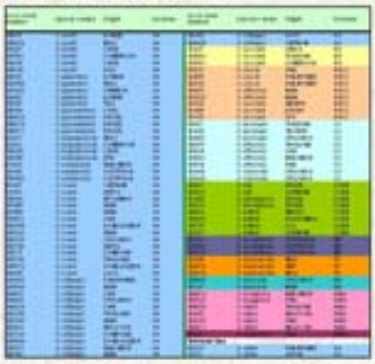
At IRRI, allele mining activities are geared towards finding novel variation at genes implicated in traits important for breeding objectives. Wild *Oryza* species have been an important source for many agronomic traits such as bacterial blight resistance (from *O. longistaminata*), grassy stunt viral resistance (from *O. nivara*), brown plant hopper resistance (from *O. australiensis*), and acid sulphate tolerance (from *O. rufipogon*). A systematic approach for characterizing wild species allelic diversity is a step towards its utilization in breeding programs.

EcoTILLING, a derivative of TILLING (Targeting Induced Local Lesions IN Genomes) was used to identify naturally occurring variation in wild *Oryza* germplasm at candidate genes involved in stress tolerance. Target loci include the drought responsive element binding protein II (DREB2), trehalose 6-phosphate phosphatase (TPP), and CG18 (14-3-3 protein), among others. Locus-specific primers were designed for upstream regulatory and coding sequence regions using the Nipponbare genomic sequence and screened using a panel representing the 22 wild *Oryza* species, and the two cultigens, *O. glaberrima* and *O. sativa*. Amplicons were generally produced in species most closely related to the cultigen (bearing the same genome, AA) and intermittently on the more distant genomes. Accessions with AA genome were contrasted against two reference lines, Nipponbare (japonica type) and IR64 (indica type). Sites of mismatches (SNPs) between heteroduplexes were cleaved with the single-strand endonuclease CEL 1 (using celery juice extract). Fluorescently labeled products were separated on denaturing PAGE using the LiCor 4300 automated genotyper. Samples prepared with unlabeled primers were ran on agarose.

The use of EcoTILLING as a diagnostic tool for biosytematics was explored. Accessions representing the AA genome *Oryza* species were subjected to heteroduplex analysis using primers for drought candidate genes. Species- specific SNP mismatches were detected and found potentially useful for defining biosystematic relationships among the AA genome *Oryza* species.

Materials and Methods

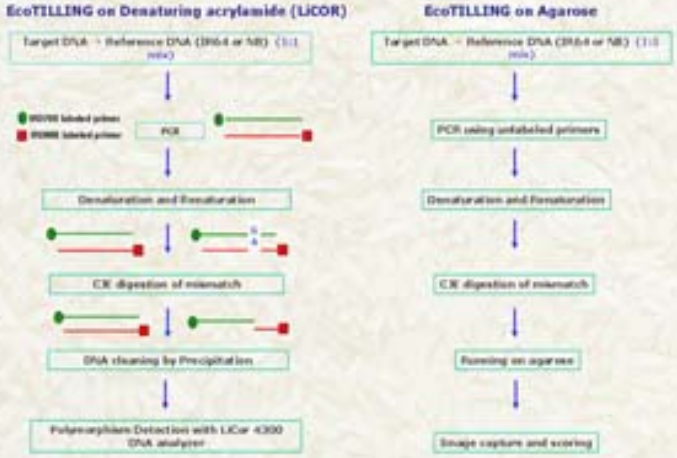
*Panel of Wild Oryza samples screened for amplicon production **



Candidate gene targets for drought tolerance

* For the biosystematic study only, wild AA genome Oryza species were included. 91 accessions were used.

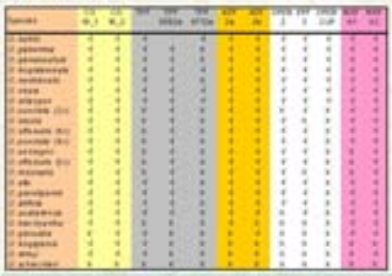
Methods of Detection



Component/Step	Acrylamide (LiCOR)	Agarose
DNA pooling	2 per pool for EcoTILLING; 8 per pool for TILLING	2 per pool for EcoTILLING; 8 per pool for TILLING
PCR primer	Labeled (IRD 700 and IRD 800) + unlabeled	Unlabeled
PCR product concentration	100 – 200 ng	400 – 500 ng
CEL 1 digestion components (per rxn)		
Celery juice extract (CJE enzyme)	0.02 ul	0.10 ul
CJE buffer	3.00 ul	1.5 ul
SUP water	16.98 ul	8.4 ul
Digestion	Add 20 ul to PCR product; digest at 45 °C for 15 mins	Add 10 ul to PCR product; digest at 45 °C for 25 mins
Post digestion steps	Stop rxn with 0.225M EDTA; mix; concentrate; load 0.3 ul sample	Stop rxn with 0.225M EDTA; load 10.0 ul sample

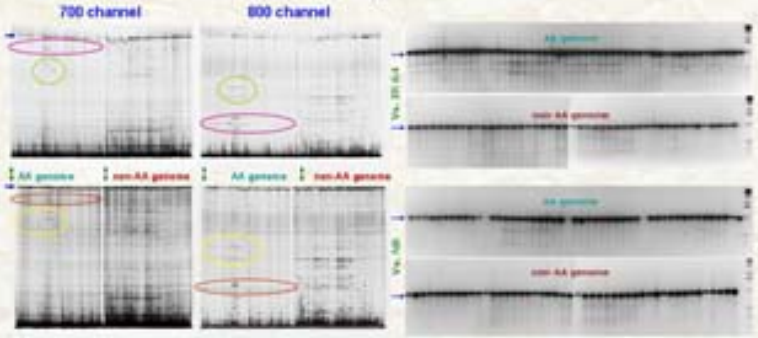
Results

Amplicons for drought candidate genes in wild Oryza



Amplicons produced in *Oryza* species for drought candidate gene primers. Generally, amplicons were produced in AA genome species and intermittently in the more distant genomes. Image at left shows amplicons obtained for the CG18-1 coding region. Products in *O. meridionalis* (red block arrow) and non-AA genome species were slightly longer than the approximately 1kb amplicon in most of the AA genome species. Two last lanes correspond to Nipponbare and IR 64 products, respectively (blue block arrow).

Mismatch pattern variation in Oryza species

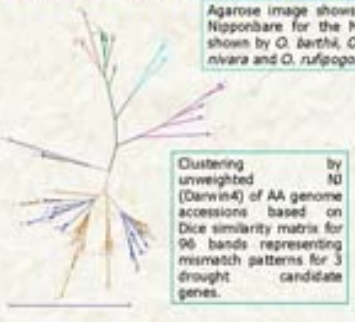


Variation in wild *Oryza* species against IR 64 and NB contrasts for the ADF2a gene detected in acrylamide (LiCOR). In the AA genome species, complementary mismatches detected in 700 and 800 channels are indicated by identically shaded circles. Among the non-AA species, mismatches are more numerous making visual scoring difficult. Full length products are indicated by blue arrow.

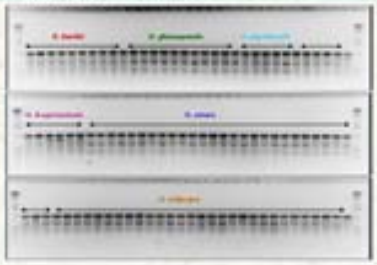
Variation in wild *Oryza* species against IR 64 and NB contrasts for the ERF3 gene detected on agarose. Among the AA genome species, mismatch patterns are more defined against the indica (IR 64) contrast where 2 to 4 mismatches are common. Among the non-AA genome species, mismatches are more numerous as with the ADF2 gene. Full length products indicated by blue arrow.

Biosystematic relationships in AA genome species defined by SNP variation

Agarose image shows mismatch patterns of the accessions contrasted against Nipponbare for the MAPk2 gene. Species-specific SNP variation patterns are shown by *O. barthii*, *O. glumaepatula*, *O. meridionalis*, and *O. longistaminata*. *O. nivara* and *O. rufipogon* show overlapping SNP patterns



Clustering by unweighted ND (Darwin4) of AA genome accessions based on Dice similarity matrix for 96 bands representing mismatch patterns for 3 drought candidate genes.



Conclusion and future work

EcoTILLING is an efficient tool for detecting SNPs in wild *Oryza* AA genome species. More primers for candidate genes for both biotic and abiotic stresses can be used against a larger set of samples to generate variation patterns that will aid in the proper utilization and conservation of wild germplasm. EcoTILLING is also potentially useful in the taxonomic classification of the AA genome species and can be used to characterize unidentified or misclassified samples.

M. E. B. Naredo, M.B. Gamalinda, G. A. Atienza, R. J. A. Melgar, and K. L. McNally
TTChang Genetic Resources Center, IRRI, DAPO Box 7777, Metro Manila 1301, the Philippines