

# 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 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 run on agarose.

The use of EcoTILLING as a diagnostic tool for biosystematics 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

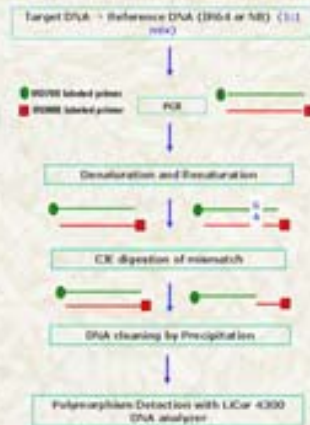
### Panel of Wild *Oryza* samples screened for amplicon production \*

Species	Accession	Genome	Type	Accession	Genome	Type
<i>O. nivara</i>	IRG725	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG726	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG727	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG728	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG729	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG730	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG731	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG732	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG733	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG734	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG735	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG736	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG737	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG738	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG739	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG740	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG741	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG742	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG743	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG744	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG745	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG746	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG747	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG748	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG749	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG750	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG751	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG752	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG753	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG754	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG755	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG756	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG757	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG758	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG759	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG760	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG761	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG762	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG763	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG764	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG765	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG766	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG767	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG768	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG769	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG770	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG771	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG772	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG773	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG774	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG775	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG776	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG777	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG778	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG779	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG780	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG781	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG782	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG783	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG784	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG785	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG786	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG787	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG788	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG789	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG790	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG791	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG792	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG793	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG794	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG795	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG796	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG797	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG798	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG799	AA	Wild	IR64	AA	Cultigen
<i>O. nivara</i>	IRG800	AA	Wild	IR64	AA	Cultigen

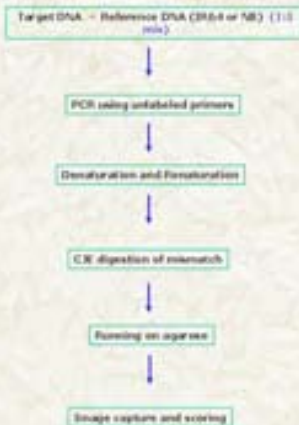
\* For the biosystematics study, only AA genome *Oryza* species were included; 96 accessions were used.

### Methods of Detection

#### EcoTILLING on Denaturing acrylamide (LICOR)



#### EcoTILLING on Agarose

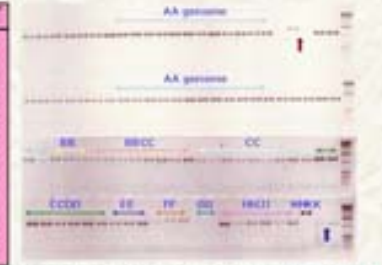


Component/Step	Acrylamide (LICOR)	Agarose
DNA pooling	2 per pool for EcoTILLING; 8 per pool for TILLING	2 per pool for EcoTILLING; 8 per pool for TILLING
PCR primer	Labeled (IRD 700 and IRD 800) + unlabeled	Unlabeled
PCR product concentration	100 – 200 ng	400 – 500 ng
CEL 1 digestion components (per 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*

Gene	IR 64	NB	IR 64	NB	IR 64	NB	IR 64	NB	IR 64	NB
ADP2										
ADP3										
ADP4										
ADP5										
ADP6										
ADP7										
ADP8										
ADP9										
ADP10										
ADP11										
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ADP14										
ADP15										
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ADP99										
ADP100										



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 CG1