Oregon State University

The Seed Laboratory

Development of Quantitative DNA Test to Distinguish between Annual and Perennial Ryegrass

Justification

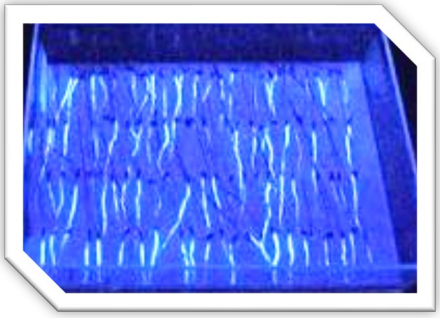
Perennial ryegrass is commonly used as turf in golf courses, home lawns, and soccer fields. Mixture of annual type in perennial type is problematic for the seed industry. The USA is the second largest exporter of ryegrass in the world *($100 M)* after Denmark *($128 M) (Trend Economy, 2021)* https://trendeconomy.com/data/commodity_h2/12092

The presence of ARG spoils the attractive look of PRG Turf.A quick, accurate DNA-based test is needed to help growers and seed companies make decisions about planting and marketing the crop.

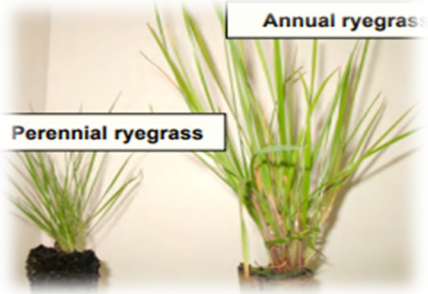
Currently, two tests are available to detect annual ryegrass in perennial samples:

1) Fluorescence test - Fluorescence trait is not always associated with the annual form of ryegrass.

2) Grow-out test - It is accurate, but it requires 6 weeks.



Roots of ARG fluoresce upon exposure to UV light.



Annual type form heads in 6 weeks, perennial do not.

Two main attempts have been made to develop a DNA test:

1. One by the Bio-Diagnostic Lab in 2011, but it was not repeatable across varieties, and it provided inconsistent results.
2. The second is the Ryegrass Allelic Discrimination (RAD) test by Reed Barker in 2014, but it has some issues:
3. Based on the fluorescence test, conducted on only seedlings that fluoresce.
4. Sample size small (only those fluoresce).
5. Takes long time (germinated and fluorescence tests first).
6. Expensive, charge per seedling.
7. Not reproducible.

Therefore, an accurate, fast, repeatable, economic, scalable test that does not depend on the fluorescent test was needed.

Objectives

Main Objective: Develop DNA test to distinguish between annual and perennial types that provides accurate, fast (48-72 hours) results and economic as well.

Specific objectives

- Screen and identify the most effective primer(s) from vernalization and flowering time genes that distinguish between annual and perennial types.
- Develop suitable protocols for DNA extraction, amplification, and detection using a Real-time PCR system.
- Perform fluorescence and grow-out tests on varieties included in the study to be compared with the PCR results, and
- Measure the repeatability and reproducibility of the new test across labs.

Studies conducted to accomplish the objectives

Study 1.

- Identify primers that best separate annual and perennial types accurately and consistently.
- Detect different levels of contamination (0-10%).

Study 2.

- Measure the reproducibility of the best primer(s) identified in study 1 across four labs.
- Replicate each contamination levels of each primer set.

Study 3.

- Assess the broader applicability and sensitivity of the test.

Study 4.

- Perform fluorescence and grow-out tests and identify the VFL values for the varieties used in the study.

Materials and Methods

- Oregon State (OSU) seed lab, USDA Utah lab, and two labs from Canada.
- OSU lab provided the seed lots.
- Four KASP primer sets were tested.
- The 4 primers sets are: Vrn2a_8, Vrn2b_2, Col1_1b, Vrn1_2.
- A PRG variety was spiked with different AGR contamination levels.
- 1 ARG in 49 PRG (50 seeds, 2%), 2 ARG in 48 PRG (50 seeds, 4%), 3 ARG in 47 PRG (50 seeds, 6%), etc.
- Three replications from each contamination level were used.

All four primers were tested at each contamination level.

The four KASP assays used in the study

Following is the summary of the four KASP assays used in the study.

Assay name	Allele X	Allele Y	CG%_X	CCG%_Y%
LolVrn2a_8	G	C	30.4	30.4
LolVrn2b_2	G	T	54.5	52.2
LolVrn1_2	G	T	37.9	34.5
LolCo1_1b	G	C	45.8	45.8

Discriptrion of the arrangements of KASP assays, different levels of ARG concentration in oerennial samples in the 96-well plate (plate map):

	1	2	3	4	5	6	7	8	9	10	11	12
	Vrn2a_8			Vrn2b_2			Col1_1b			Vrn1_2		
A	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG	ARG
B	PRG	PRG	PRG	PRG	PRG	PRG	PRG	PRG	PRG	PRG	PRG	PRG
C (2%)	Spike 2%	Spike 2%	Spike 2%	Spike 2%	Spike 2%	Spike 2%	Spike 2%	Spike 2%	Spike 2%	Spike 2%	Spike 2%	Spike 2%
D (4%)	Spike 4%	Spike 4%	Spike 4%	Spike 4%	Spike 4%	Spike 4%	Spike 4%	Spike 4%	Spike 4%	Spike 4%	Spike 4%	Spike 4%
E (6%)	Spike 6%	Spike 6%	Spike 6%	Spike 6%	Spike 6%	Spike 6%	Spike 6%	Spike 6%	Spike 6%	Spike 6%	Spike 6%	Spike 6%
F (8%)	Spike 8%	Spike 8%	Spike 8%	Spike 8%	Spike 8%	Spike 8%	Spike 8%	Spike 8%	Spike 8%	Spike 8%	Spike 8%	Spike 8%
G (10)	Spike 10%	Spike 10%	Spike 10%	Spike 10%	Spike 10%	Spike 10%	Spike 10%	Spike 10%	Spike 10%	Spike 10%	Spike 10%	Spike 10%
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Following is the protocol used in the DNA extraction, amplification, and the Real-time PCR test:

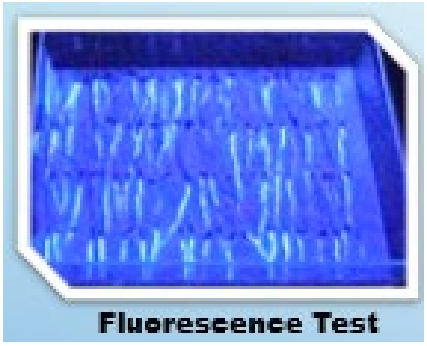
	1 RXN (µL per well)	
DNA 2.5 ng/µL	5	
2x KASP-TF Master Mix	5	
KASP Assay Mix (Primers)	0.14	
Water	N//A	
Total volume	10.14	

	Step 1	Step 2	Step 3	Step 4
Thermocyler	Hold	PCR Stage	PCR stage	Post read
		10 cycles	26 cycles	1 cycle
	94°C for 15 min.	94°C for 20 sec.	94°C for 20 sec.	30°C (read)
		61°C decreasing 0.6°C per cycle for 60 sec.	55°C for 60 sec. (read)	

RESULTS

Standard Germination, Fluorescent, and Grow-out Test Results of Varieties Used in the PCR Test

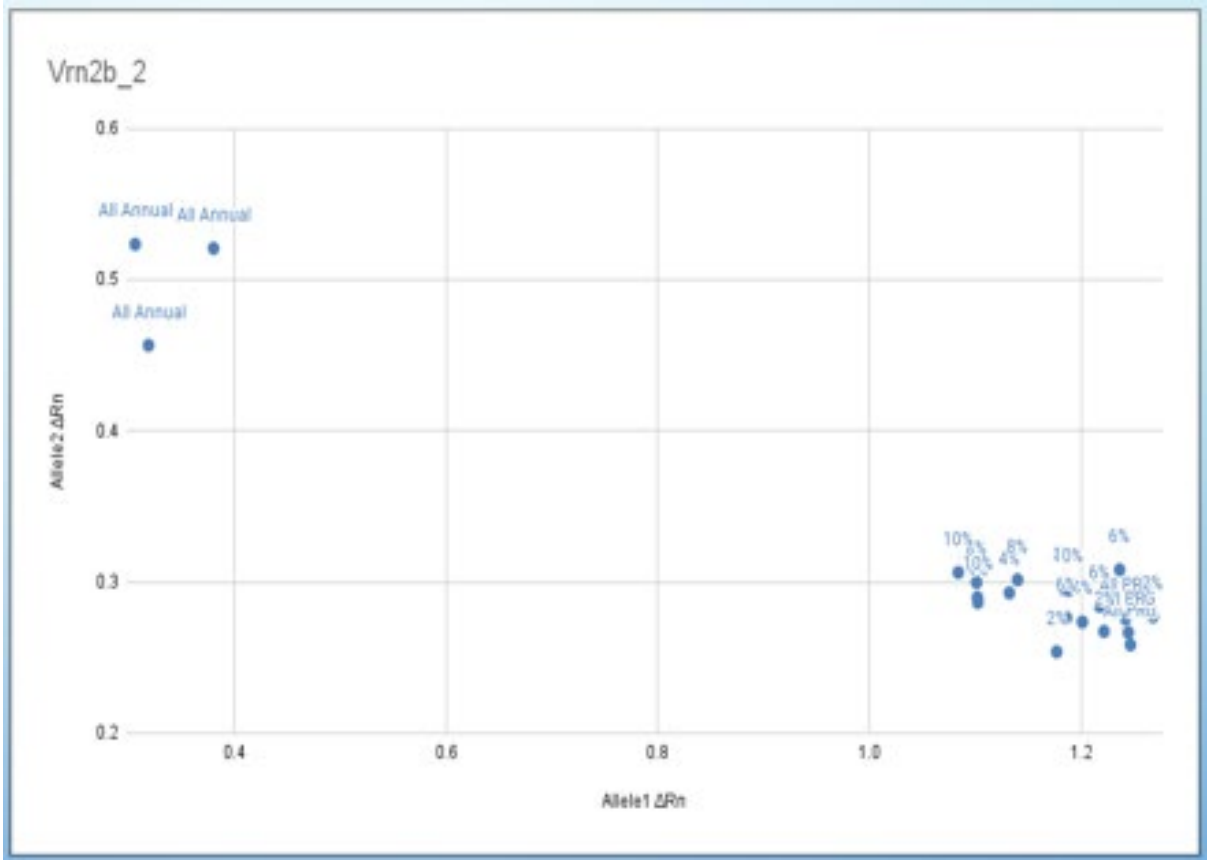
Crop	Sample ID	Germination (%)	Fluorescence (%)	Transplanted	PRG	Grow-out (%)
ARG	1	95	99.21	N/A	N/A	100
	2	96	100.00	N/A	N/A	100
	3	98	100.00	N/A	N/A	100
	4	92	100.00	N/A	N/A	100
	5	95	99.74	N/A	N/A	100
	6	97	100.00	N/A	N/A	100
PRG	1	79	0.95	3	3	0
	2	82	0.61	2	2	0
	3	94	0.27	1	1	0
	4	90	0.00	0	0	0
	5	18	0.00	0	0	0
	6	90	0.28	1	1	0



The fluorescence and grow-out tests produced similar results to the controls of the KASP assays.

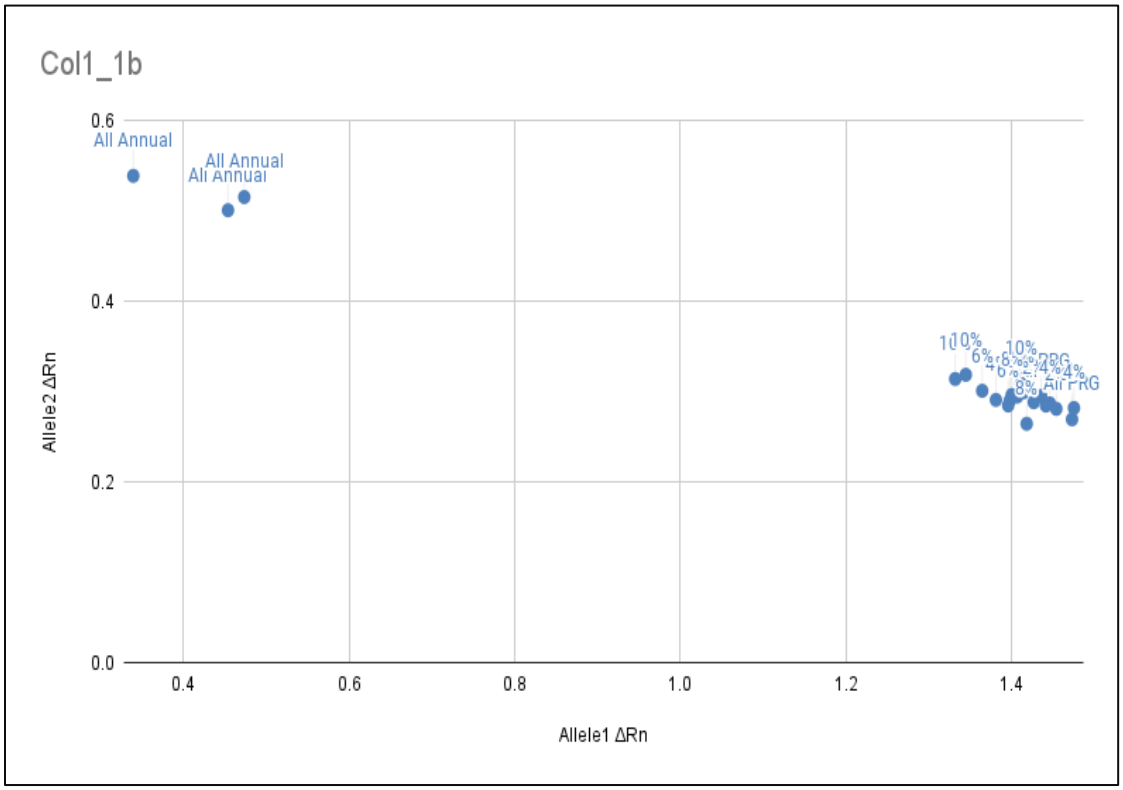
Summary of the Real-time PCR runs of the four KASP assays:

1. The first KASP set - Vrn2b_2 Results



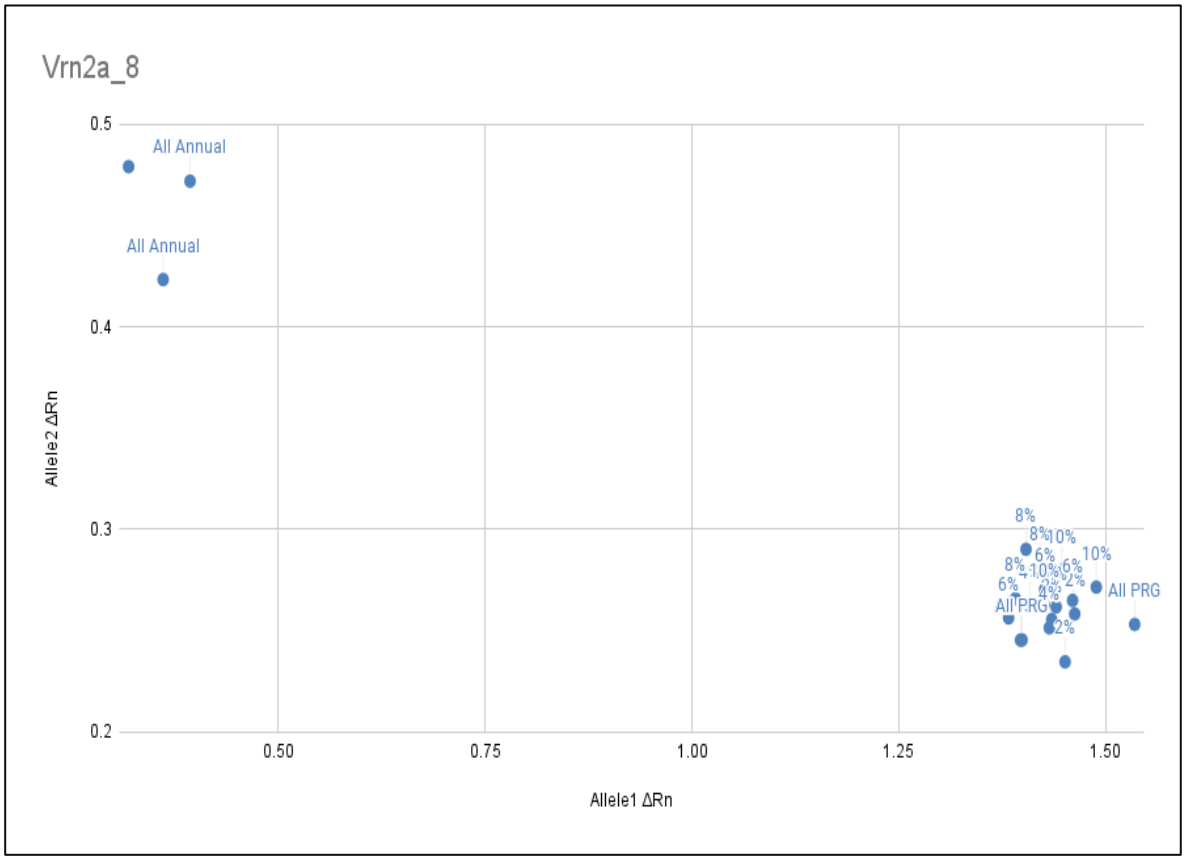
- Pure annual and pure perennial are where they should be.
- Quantification of contamination is difficult.

2. The second KASP set - Col1_1b Results



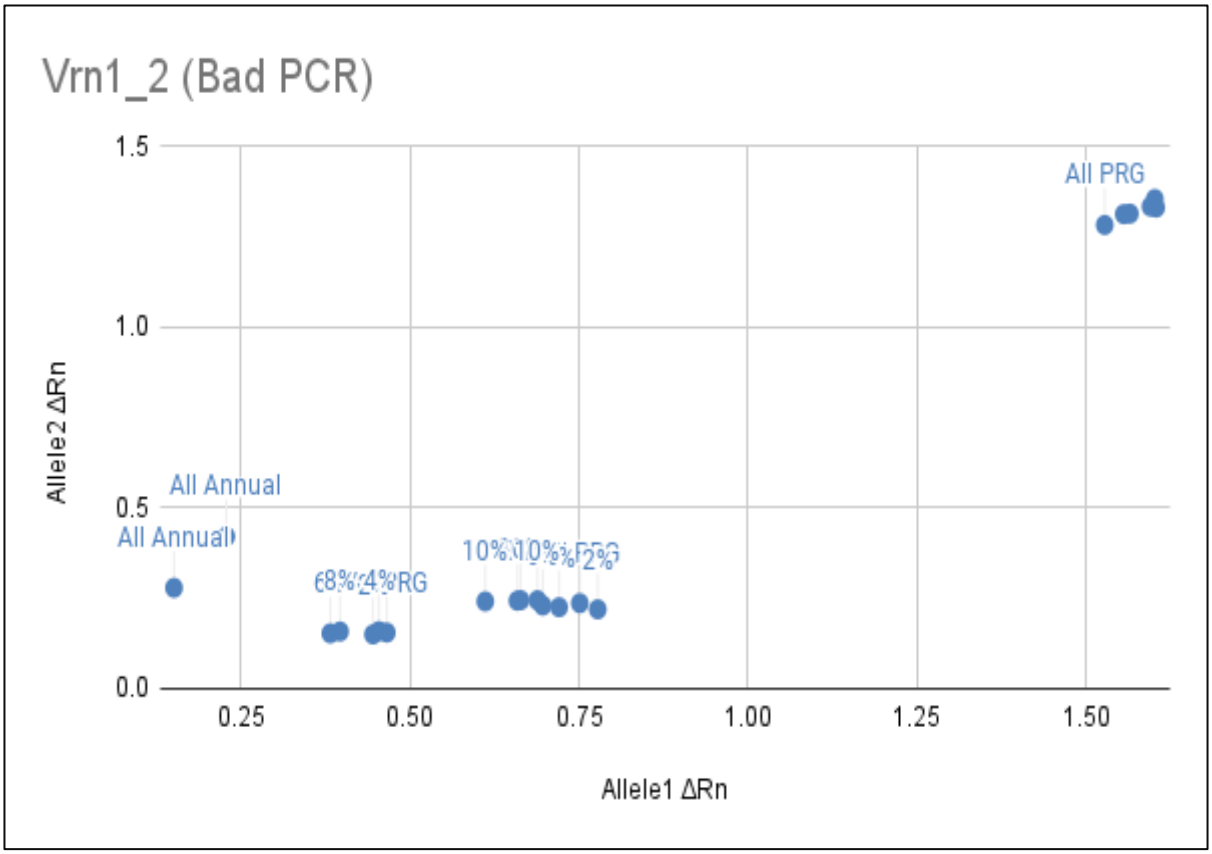
- Pure annual and pure perennial are where they should be.
- Similar results to Vrn2b_2

3. The third KASP set - Vrn2a_8 Results



- Pure annual and pure perennial are where they should be.
- Vrn2b_2 and Col1_1b are likely good primer sets, after the first two assays.

4. The fourth KASP set - Vrn1_2 Results



- Pure annual and pure perennial are not where they should be.
- Odd clustering behavior.

Conclusions

- Primers that distinguish between ARG and PRG were identified.
- Vrn2b_2, and Col1_1b were found to be better primer sets than Vrn2a_8; and Vrn1_2 was not suitable for the assay.
- KASP real-time PCR protocol was developed to detect ARG contamination in PRG seed samples.
- The reproducibility of the test was validated across four labs.
- The new developed test can detect annual contamination up to 10% in perennial samples.
- Fluorescence and the grow-out tests were conducted.
- Downside: KASP analysis is not sensitive enough for quantifying low levels of annual contamination in PRG samples (0.1%). However, it has useful applications in detecting contamination in breeders, foundation, certified and other samples that do not require very small level of contamination.
- Future study: genotyping by sequencing (GBS), a qPCR method for accurate quantification of annual contamination.

Acknowledgements

- Special thanks to the Seed Testing Research Foundation (STRF) for the partial financial support of this project.
- Thanks to the USDA Lab in Utah for the collaboration in this project.
- Thanks to ISTA for the cooperation in this project and partial support.
- Thanks to the Canadian labs that participated in the project.