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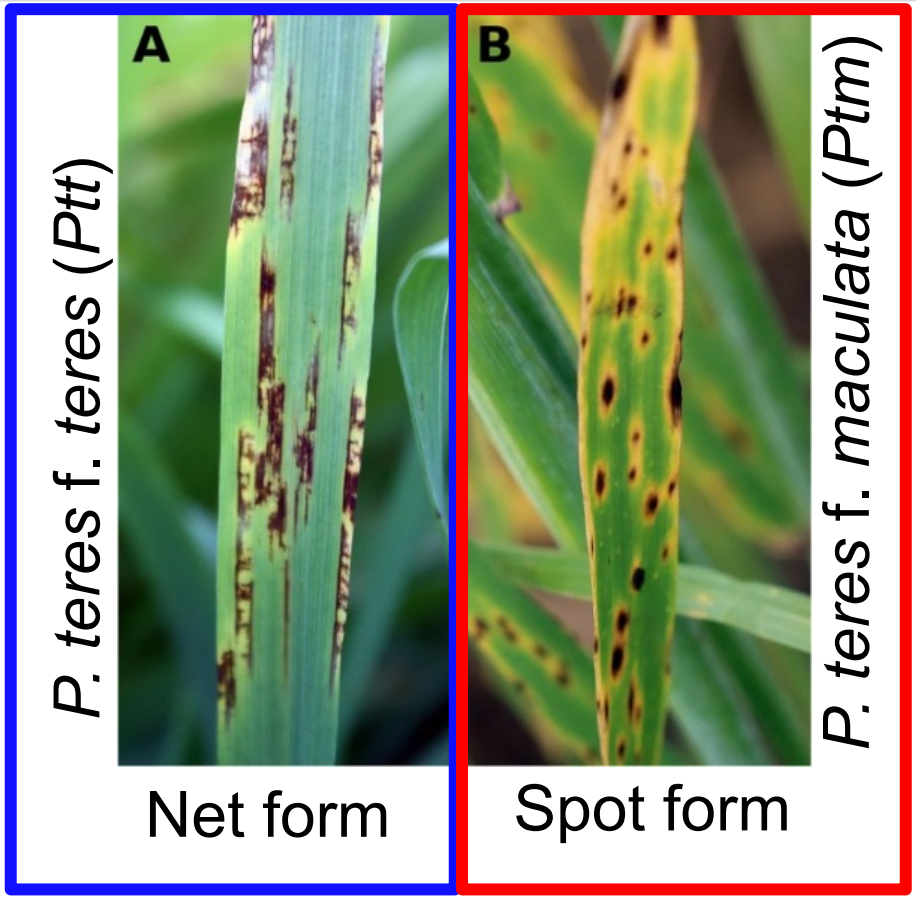
Parallel evolution of fungicide resistance in the barley net blotches:

EMERGENCE OF A RESISTANT CLONAL POPULATION BY INTERSPECIFIC HYBRIDISATION

Wesley Mair, Curtin University

Net Blotches of Barley – *Pyrenophora teres*



- Among the most economically significant diseases of barley (*Hordeum vulgare*) worldwide.
- Two forms which are closely related, but genetically isolated and considered two distinct species (Ellwood *et al*. 2012):
  - *P. teres* f. sp. *teres* (*Ptt*), Net-form of Net blotch (NFNB)
  - *P. teres* f. sp. *maculata* (*Ptm*), Spot-form of Net blotch (SFNB)
- Demethylase-inhibitor (DMI) fungicides are a key component of control programs

Syme, RA, Martin, A, Wyatt, NA, Lawrence, JA, Muria-Gonzalez, MJ, Friesen, TL, Ellwood, SR (2018) Transposable Element Genomic Fissuring in Pyrenophora teres Is Associated With Genome Expansion and Dynamics of Host–Pathogen Genetic Interactions. *Frontiers in Genetics* **9**, 130

Ellwood, SR, Syme, RA, Moffat, CS, Oliver, RP (2012) Evolution of three Pyrenophora cereal pathogens: Recent divergence, speciation and evolution of non-coding DNA. *Fungal Genetics and Biology* **49**, 825-829.

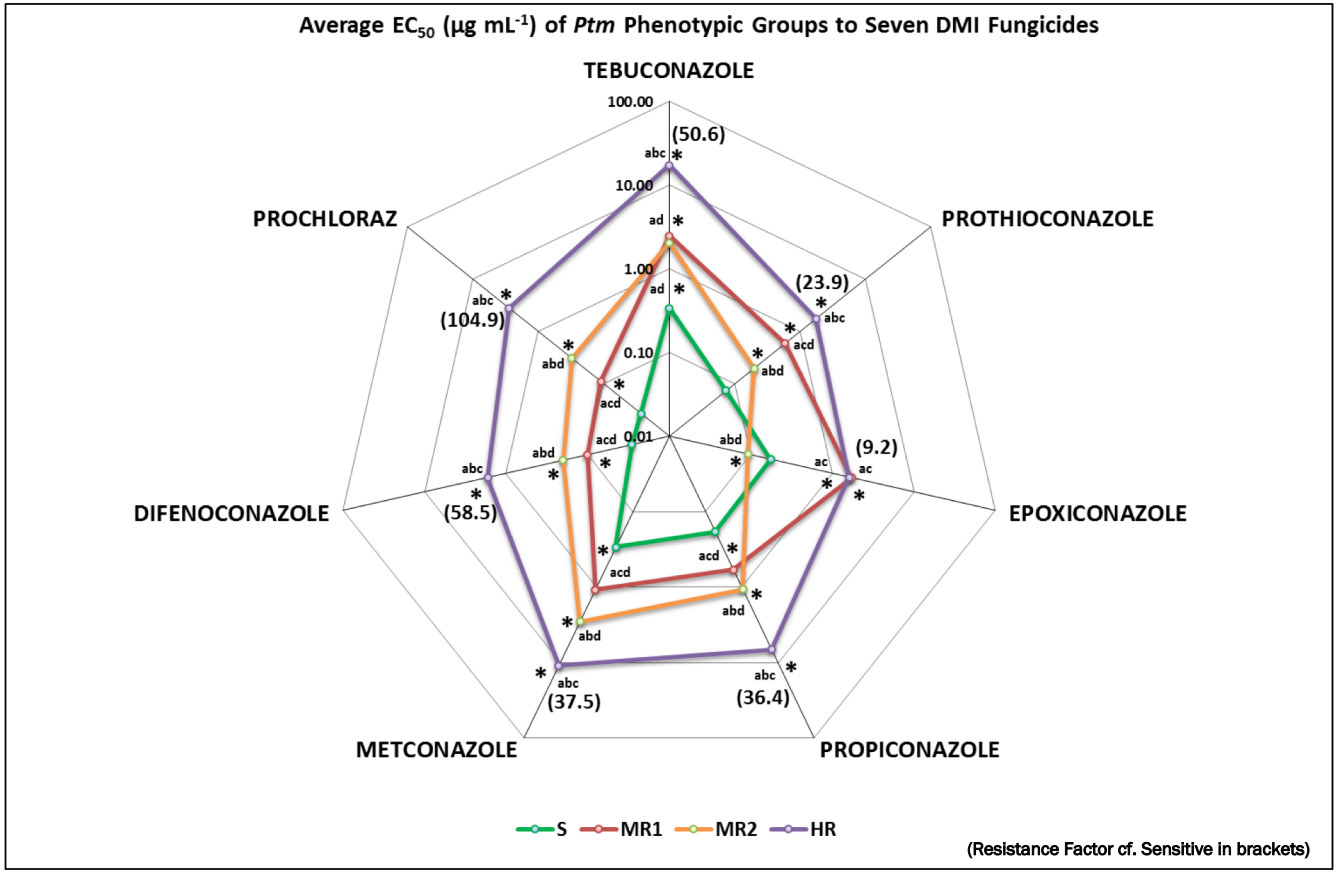
Shock & Awe, or: When Control Fails

Photographs courtesy of Kith Jayasena (Department of Primary Industries & Regional Development, Western Australia).

A field in Western Australia (WA) showing symptoms of SFNB infection, following treatment with:

1. Tebuconazole, 400mL/100kg (SD)
2. Propiconazole, 325mL/ha @ Z25
3. Cyproconazole + Azoxystrobin, 400mL/ha @ Z31
4. Epoxiconazole, 250mL/ha @ Z39
5. Propiconazole, 500mL/ha @ Z52

DMI sensitivity phenotypes in *Ptm*



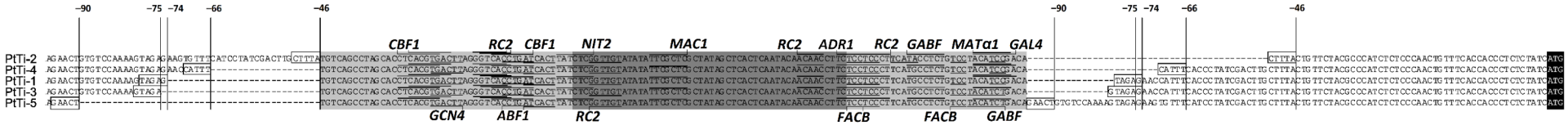
*The mean difference between groups [a]S, [b]MR1, [c]MR2 & [d]HR is significant at 0.05 level (Kruskal-Wallis H test & Dunnett's T3)

In vitro analysis of 288 isolates of *Ptm* collected 1996-2019 from WA barley-growing regions:

- Sensitive (S) phenotype
- Two Moderately-Resistant (MR) phenotypes:
  - MR1
  - MR2
- Highly-Resistant (HR) phenotype

Five insertions in the *Cyp51A* promoter

- Insertions of 134-bp were found at five different sites upstream of the gene encoding the DMI target (*Cyp51A*), in MR1 & HR *Ptm*:
  - At –66, –74, or –75 in MR1 isolates
  - At –46 or –90 in HR isolates



Light grey box: sequences homologous to LTRs of *Ty1/Copia* retrotransposons; Dark grey box: predicted promoter sequences; White box: putative TSD sequences; Black box: start codons; Underlined/overlined: predicted transcription factor binding sites.

- 129-bp homologous to Long Terminal Repeats (LTRs) of *Ty1/Copia*-family retrotransposons
  → Solo-LTR elements formed through unequal homologous recombination (Devos *et al*. 2002)
- Insertion sequences contained predicted promoter and transcription factor binding sites

Devos, KM, Brown, JKM, Bennetzen, JL (2002) Genome size reduction through illegitimate recombination counteracts genome expansion in Arabidopsis. Genome Research **12**, 1075-1079

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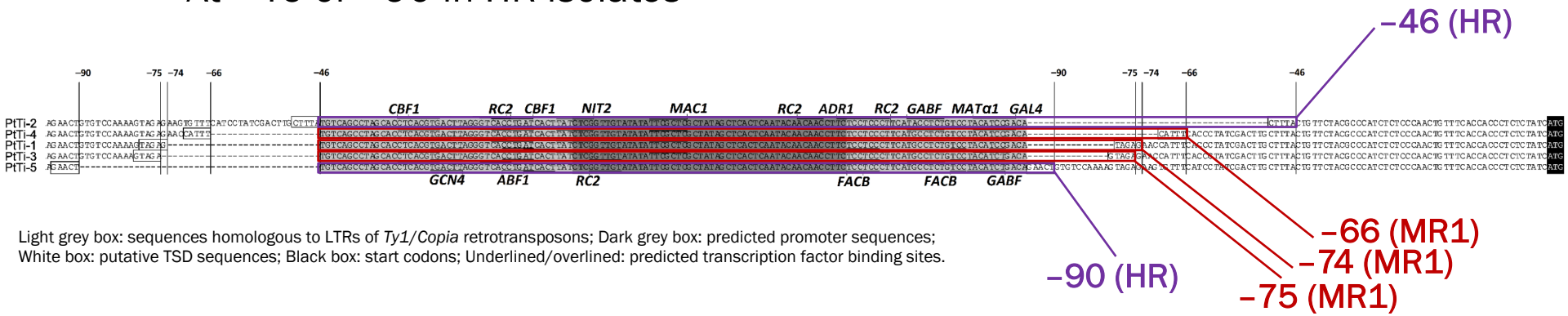

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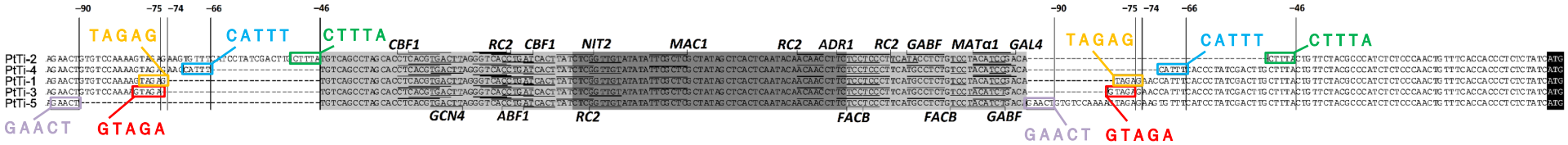
Multiple integrations of transposons

- Each of the five insertion elements are flanked by distinct 5-bp direct repeat sequences
- Target Site Duplications occuring as a result of staggered double-stranded DNA breaks at the occasion of transposon integration (Craig 2002)
  - Characteristically result in 4-6 bp flanking direct repeats for insertions of LTR transposons (Wicker *et al*. 2007)



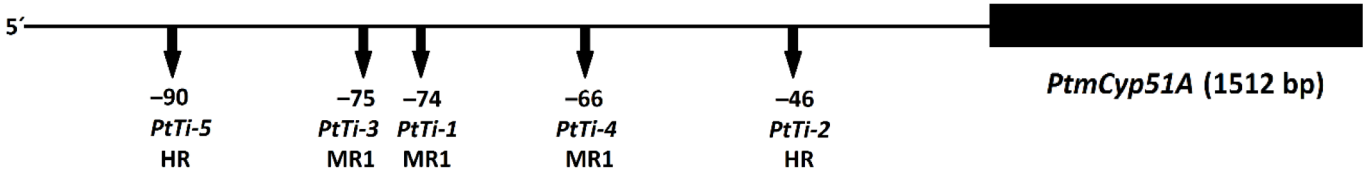
Light grey box: sequences homologous to LTRs of *Ty1/Copia* retrotransposons; Dark grey box: predicted promoter sequences; White box: putative TSD sequences; Black box: start codons; Underlined/overlined: predicted transcription factor binding sites.

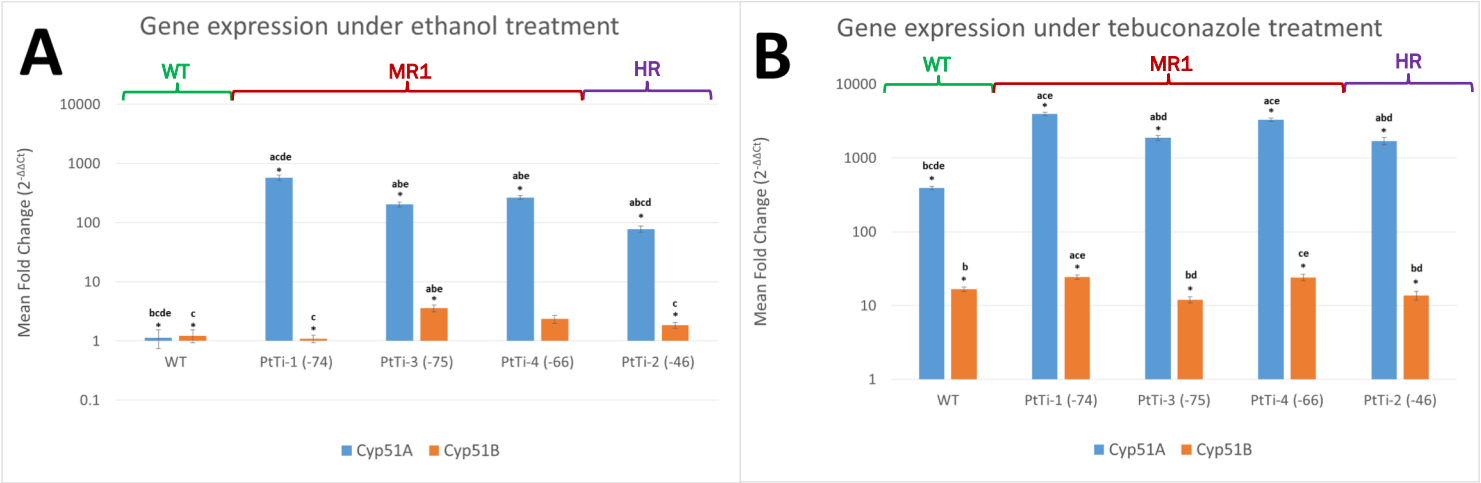
- The insertions also appear in backgrounds of distinct haplotypes of the *Cyp51A* promoter region
- Indicate multiple discrete insertion events of *Ty1/Copia* LTR retrotransposons

Craig, Nancy L.. 2002. Mobile DNA: an Introduction, p 3-11. In: Craig, Nancy L. and Craigie, Robert and Gellert, Martin and Lambowitz, Alan M.(ed), 'Mobile DNA II'. American Society of Microbiology

Wicker, T, Sabot, F, Hua-Van, A, Bennetzen, JL, Capy, P, Chalhoub, B, Flavell, A, Leroy, P, Morgante, M, Panaud, O, Paux, E, SanMiguel, P, Schulman, AH (2007) A unified classification system for eukaryotic transposable elements. Nat Rev Genet **8**, 973-82.

Constitutive overexpression of *Cyp51A*





- Insertions contain predicted promoter & transcription factor binding sites
- Presence of insertion correlated with *Cyp51A* over-expression (in both presence & absence of DMIs)

*The mean difference between isolates [a]M2, [b]16FRG073, [c]17FRG178, [d]18FRG003, and [e]17FRG089 is significant at the 0.05 level (Kruskal-Wallis H test & Dunnett's T3)

Three different SNPs for F489L in CYP51A

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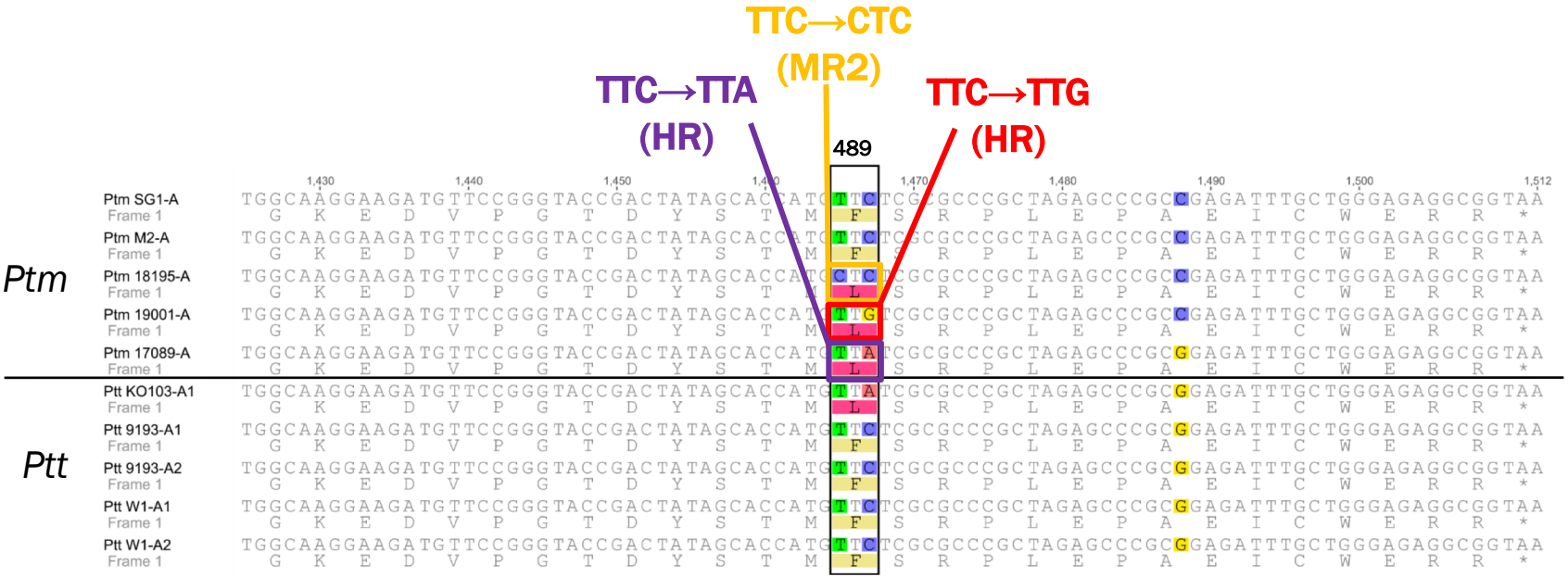
- F489L substitution in CYP51A (corresponding to *F495I* in archetype sequence of *Aspergillus fumigatus*, Mair *et al*. 2016) was found in HR & MR2 *Ptm*
- Amino acid change from either of three different SNPs in codon 489:



Mair, W, Lopez-Ruiz, F, Stammler, G, Clark, W, Burnett, F, Hollomon, D, Ishii, H, Thind, TS, Brown, JK, Fraaije, B, Cools, H, Shaw, M, Fillinger, S, Walker, A-S, Mellado, E, Schnabel, G, Mehl, A, Oliver, RP (2016) Proposal for a unified nomenclature for target-site mutations associated with resistance to fungicides. Pest management science **72**, 1449-1459.

Three mutational events for F489L (*F495*)

- F489L substitution in CYP51A (corresponding to *F495I* in archetype sequence of *Aspergillus fumigatus*, Mair *et al*. 2016) was found in HR & MR2 *Ptm*
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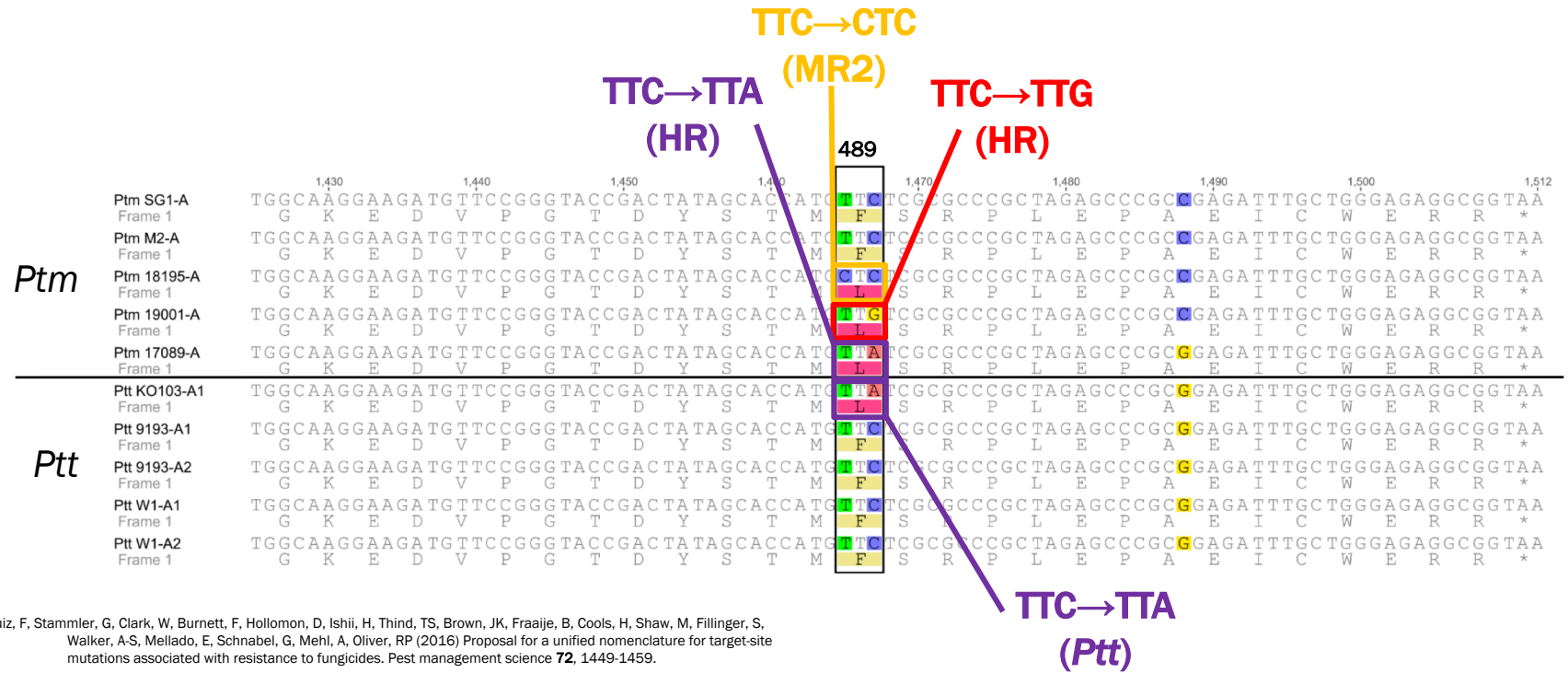

Mair, W, Lopez-Ruiz, F, Stammler, G, Clark, W, Burnett, F, Hollomon, D, Ishii, H, Thind, TS, Brown, JK, Fraaije, B, Cools, H, Shaw, M, Fillinger, S, Walker, A-S, Mellado, E, Schnabel, G, Mehl, A, Oliver, RP (2016) Proposal for a unified nomenclature for target-site mutations associated with resistance to fungicides. Pest management science **72**, 1449-1459.

F489L (*F495*) mutation of CYP51A in *Ptt*

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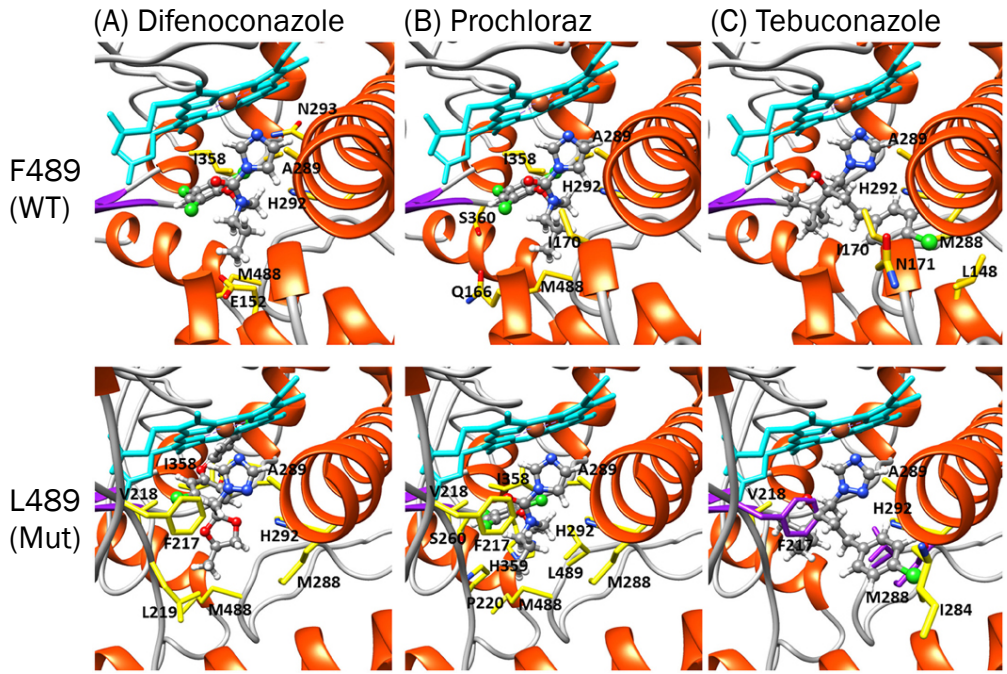
The TTC⟶TTA mutation for F489L has also been previously reported in *P. teres* f. *teres* with reduced sensitivity to DMIs (Mair *et al*. 2016):



Fungicides in ball and stick, heme groups in cyan, showing the location of each azole and interacting residues shown in yellow and labeled.

Residues within 3 Å of the docked azoles and predicted binding affinities (kcal mol^{-1}):

	Difenoconazole	Prochloraz	Tebuconazole
F489 (WT)	E152, **A289**[a], **H292**, N293, N356, A357, I358	Q166, I170, **A289**, **H292**, A357, I358, H359, S360, M488, F489	L148, I170, N171, M288, **A289**, **H292**
Binding affinity	−10.600	−9.100	−9.500
L489 (Mut)	F217, V218, L219, **M288**, **A289**, **G290**, **H292**, N356, A357, I358, M488	F217, V218, P220, **M288**, **A289**, **G290**, **H292**, A357, I358, H359, S360, M488, L489	F217, V218, I284, **M288**, **A289**, **G290**, **H292**, L489
Binding affinity	−9.100	−7.500	−8.200[b]

[a] *Residues in bold are part of the M288-H292 helical region of the binding cavity that are found in close proximity to the azole-heme complex.*
[b] *Predicted binding affinity based on alternative docking location.*

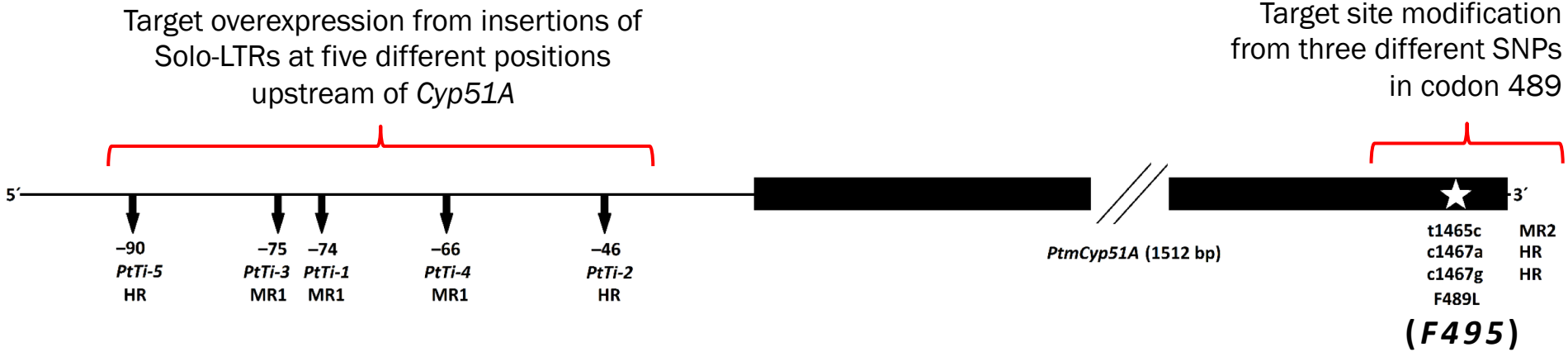
Mair, WJ, Deng, W, Mullins, JG, West, S, Wang, P, Besharat, N, Ellwood, SR, Oliver, RP, Lopez-Ruiz, FJ (2016b) Demethylase Inhibitor Fungicide Resistance in Pyrenophora teres f. sp. teres Associated with Target Site Modification and Inducible Overexpression of Cyp51. Front Microbiol **7**, 1279.

Mechanisms of DMI resistance in *Ptm*

- Moderately DMI-resistant isolates have one or the other of these mechanisms:
  - MR1 ⟶ 134-bp insert only ⟶ Three distinct genotypes

    or
  - MR2 ⟶ F489L only
- Highly DMI-resistant (HR) isolates have combinations of both mechanisms (134-bp insert + F489L)

  ⟶ Two distinct genotypes with different insertion sites & SNPs in codon 489



A hybrid origin?

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- Has the TTC⟶TTA mutation for F489L in *Ptm* introgressed from DMI-resistant *Ptt*?
- Inter-form hybrids deriving from sexual reproduction between *Ptt* & *Ptm* have been reported in the field at low frequencies (eg. Campbell *et al*. 2002; Leišova *et al*. 2005; McLean *et al*. 2014; Poudel *et al*. 2017)
- ‘Markers which clearly distinguish the two forms of *P. teres* and enable unambiguous identification of hybrids’ (Poudel *et al*. 2017).

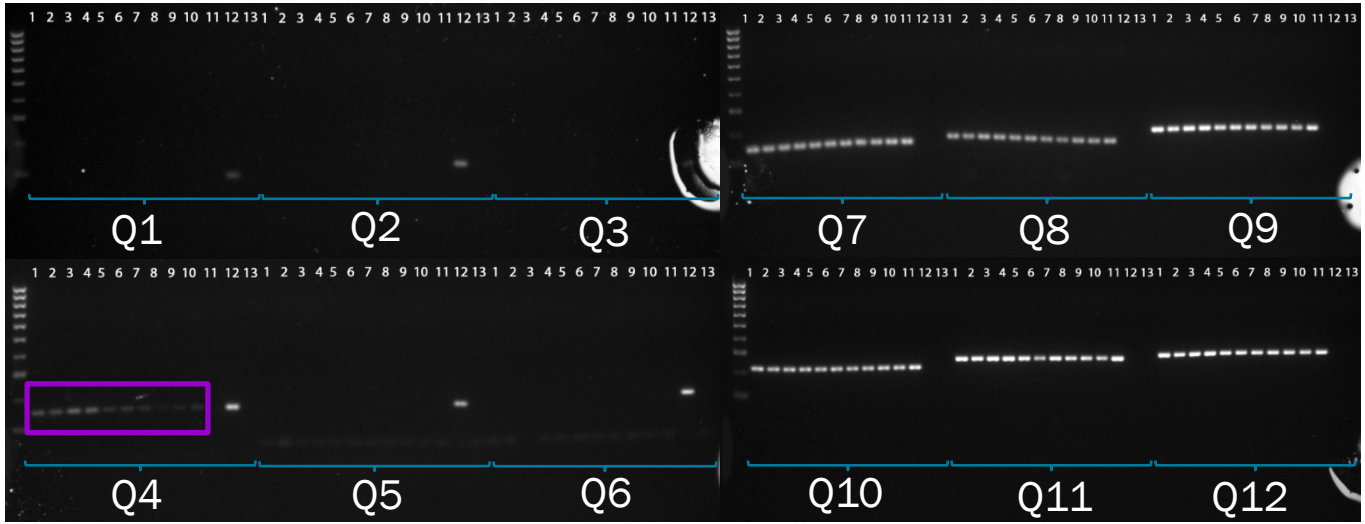
Campbell, GF, Lucas, JA, Crous, PW (2002) Evidence of recombination between net- and spot-type populations of Pyrenophora teres as determined by RAPD analysis. Mycological Research **106**, 602-608.
Leišova, L, Minarˇkova, V, Kučera, L, Ovesná, J (2005) Genetic Diversity of Pyrenophora teres Isolates as Detected by AFLP Analysis. Journal of Phytopathology **153**, 569-578.
McLean, M, Martin, A, Gupta, S, Sutherland, M, Hollaway, G, Platz, G (2014) Validation of a new spot form of net blotch differential set and evidence for hybridisation between the spot and net forms of net blotch in Australia. Australasian Plant Pathology **43**, 223-233.
Poudel, B, Ellwood, SR, Testa, AC, McLean, M, Sutherland, MW, Martin, A (2017) Rare Pyrenophora teres Hybridization Events Revealed by Development of Sequence-Specific PCR Markers. Phytopathology **107**, 878-884.

c1467a mutants carry *Ptt* & *Ptm* markers

- All HR *Ptm* with F489L (c1467a) mutation are positive for 6/6 *Ptm*, 1/6 *Ptt* form-specific markers (*Ptt*Q4)
- Identical pattern observed across >50 HR isolates

Ptt markers:

Ptm markers:



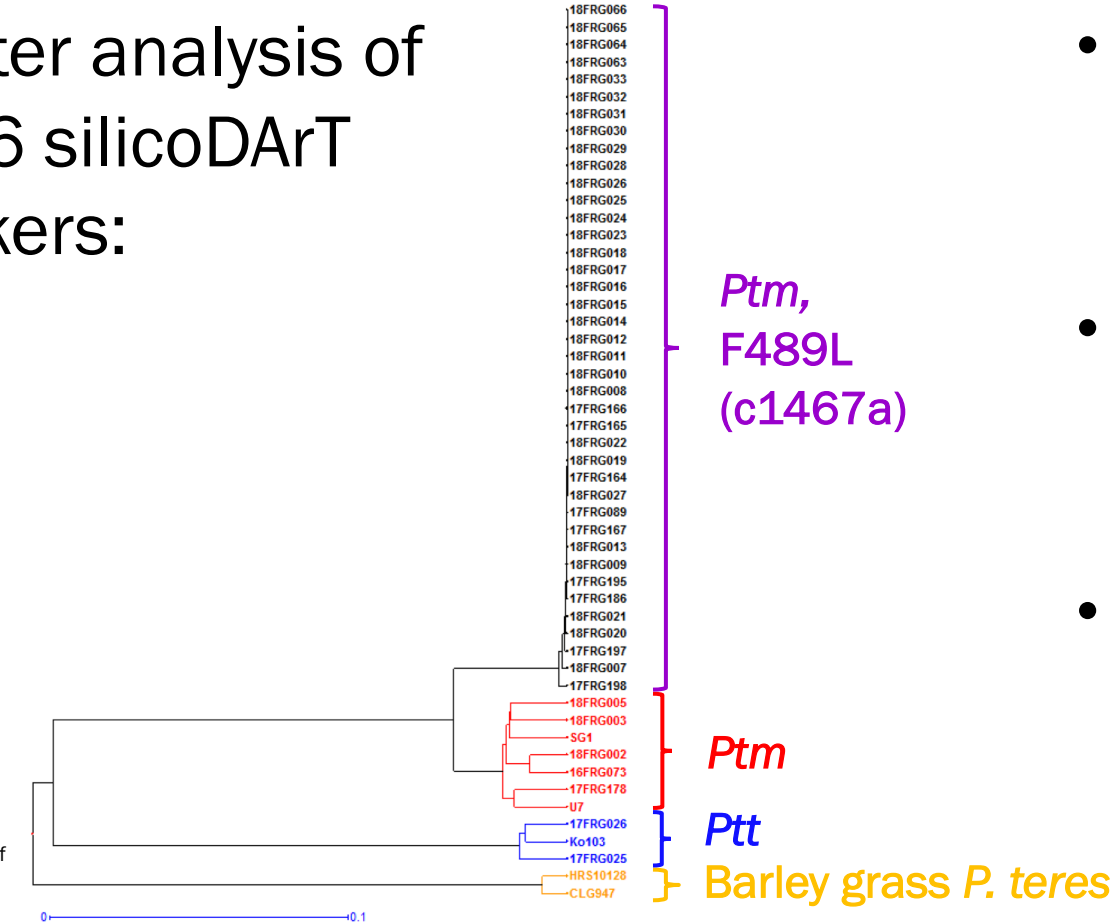
Lane
1. 17FRG164
2. 17FRG165
3. 17FRG166
4. 17FRG167
5. 17FRG186
6. 17FRG187
7. 17FRG195
8. 17FRG196
9. 17FRG197
10. 17FRG198
11. SG1 (*Ptm* Control)
12. Ko103 (*Ptt* Control)
13. No Template Control

F489L (c1467a) HR *Ptm*

2% Agarose-TAE, 100 V × 90 min

c1467a mutants distinct from *Ptm* & *Ptt*

Cluster analysis of 9656 silicoDArT markers:

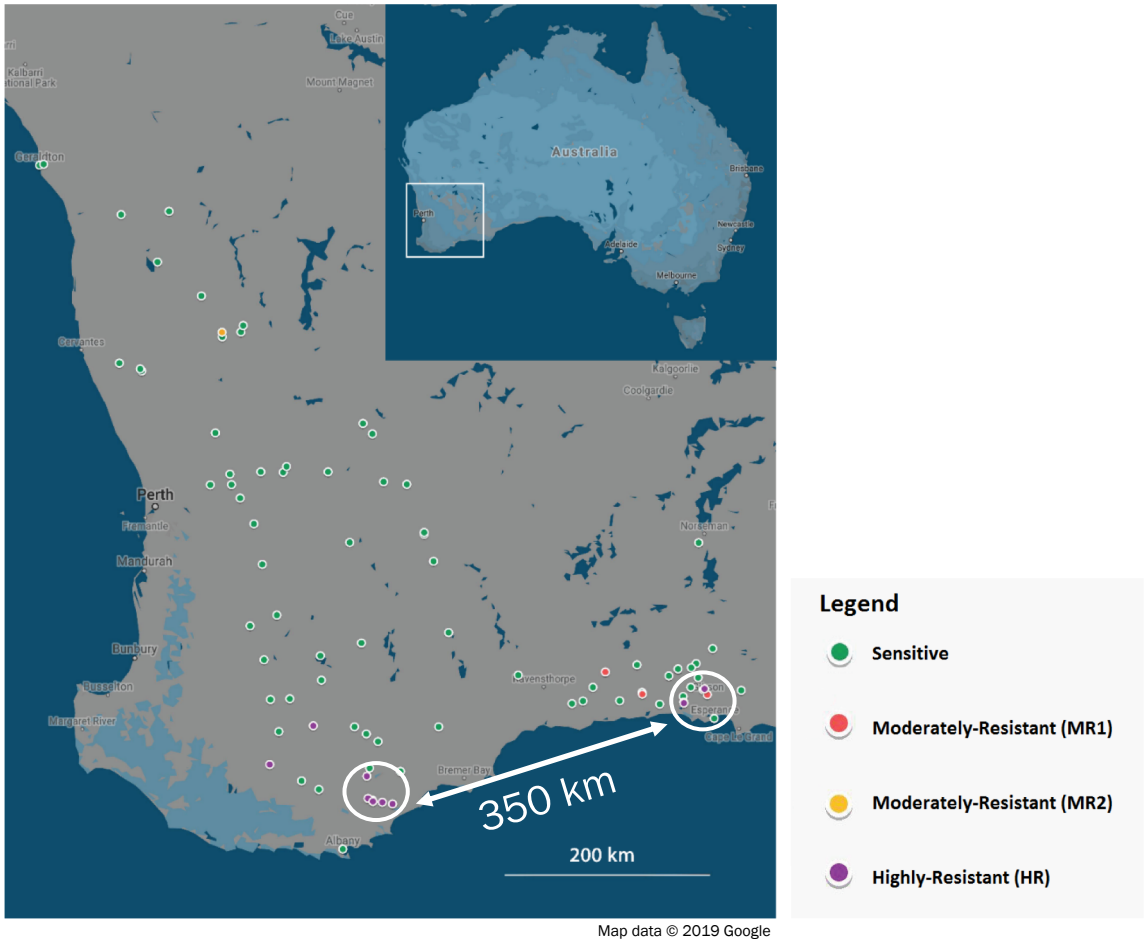


Similarity matrix constructed with DICE coefficient, cluster analysis of matrix values by UPGMA.

- Do not cluster with *Ptt* isolates or *P. teres* isolates from barley grass
- Do not cluster with *Ptm* isolates but are closely related to them
- Lack of genetic diversity among 40 HR isolates tested
  - Majority are clonal

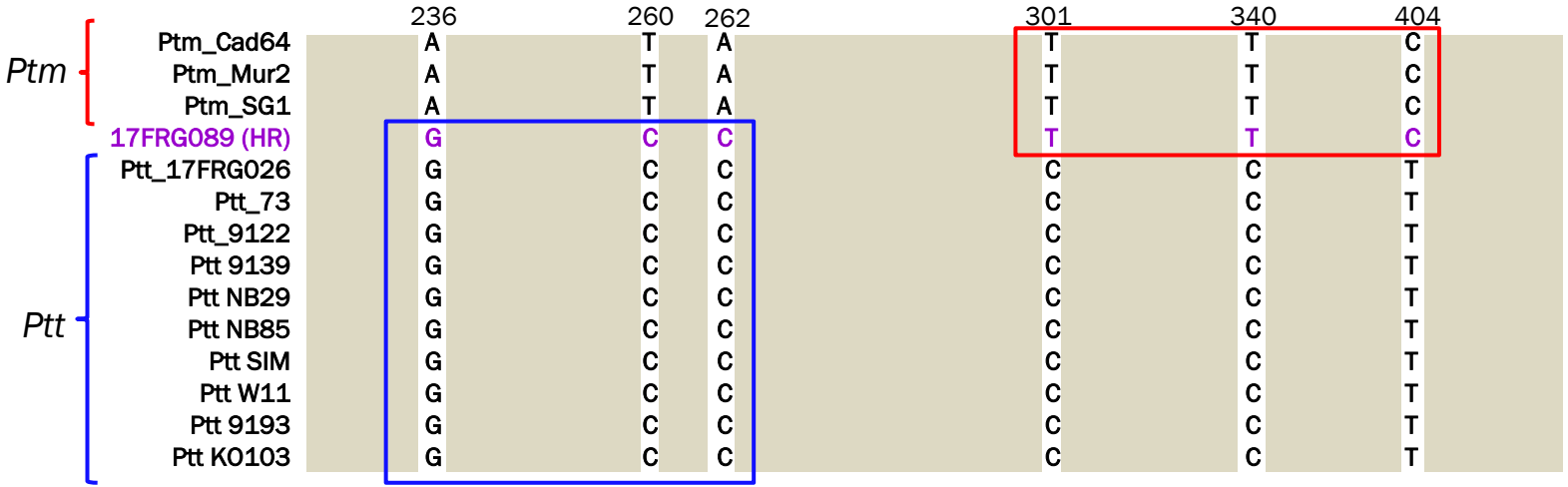
Attack of the clones

Clonal isolates were collected from 7 locations across the barley growing regions of Western Australia, separated by distances of up to 350 km



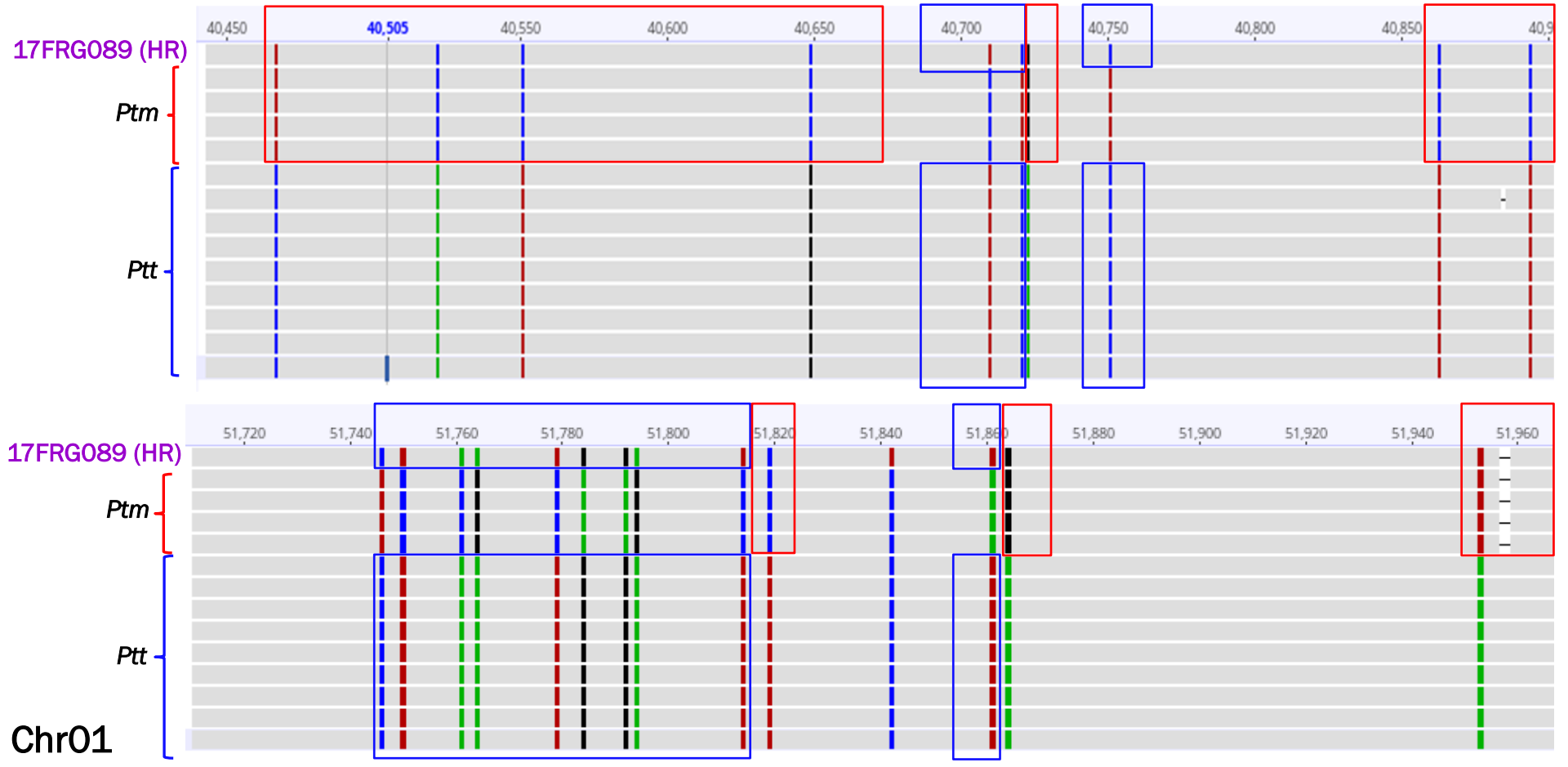
Footprint of recombination with *Ptt*

- Genome-wide population genetics and phylogenetic analysis to address evidence of recombination between *Ptt* & *Ptm*
- Analysis of genomes of nine *Ptt* and five *Ptm* isolates (including one HR)
- Alignment of intergenic regions:
  - Select orthologous regions conserved across all 14 genomes, >0.5kbp from any genes, filter any regions overlapping with predicted repeats



Chr06

Consistent SNP patterns observed



Intergenic regions showing recombination

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Chr (W11)	# regions tested	# recombinations detected	*P* values (Φ_w)
Chr01	202	7	0.000 – 0.0234
Chr02	226	12	0.0004 – 0.008
Chr03	143	10	0.0003 – 0.042
Chr04	135	2	0.000 – 0.030
Chr05	153	7	0.0004 – 0.031
Chr06	113	10	0.000 – 0.035
Chr07	85	4	0.000 – 0.009
Chr08	84	6	0.000 – 0.036
Chr09	61	5	0.000 – 0.024
Chr10	83	6	0.000 – 0.023
Chr11	43	6	0.000 – 0.014
Chr12	65	0	NS
Total	1393	75	

- Pairwise Homoplasy Index (PHI, Φ_w) test: nonparametric test for detecting the presence of recombination (Bruen *et al*., 2006)
- Among 1393 intergenic regions tested, 75 suggested presence of significant ($P < 0.05$) recombination.

Bruen, TC, Philippe, H, Bryant, D (2006) A Simple and Robust Statistical Test for Detecting the Presence of Recombination. Genetics **172**, 2665-2681.

Conclusions

- Multiple insertion events of *Ty1/Copia* LTR retrotransposons upstream of *Cyp51A* in *Ptm*
- Target site modification (F489L) in CYP51A has emerged in both *Ptt* and *Ptm*, from at least 3 distinct mutational events
- HR *Ptm* carrying the SNP for F489L identical to *Ptt* (c1467a) also shows evidence of recombination between the two forms
- Minimal genetic diversity among HR c1467a *Ptm* isolates found across the barley growing regions of Western Australia
- Emergence of HR strain in WA likely arose from hybridisation of *Ptm* & *Ptt*, followed by backcrossing to *Ptm* & clonal dispersion

Acknowledgements

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