**Review**

Elusive Diagnostic Markers for Russian Wheat Aphid Resistance in Bread Wheat: Deliberating and Reviewing the Status Quo

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**Abstract:** Russian wheat aphid, *Diuraphis noxia* (Kurdjumov), is a severe pest of wheat, *Triticum aestivum* L., throughout the world. Resistant cultivars are viewed as the most economical and environmentally viable control available. Studies to identify molecular markers to facilitate resistance breeding started in the 1990s, and still continue. This paper reviews and discusses the literature pertaining to the *D. noxia* R-genes on chromosome 7D, and markers reported to be associated with them. Individual plants with known phenotypes from a panel of South African wheat accessions are used as examples. Despite significant inputs from various research groups over many years, diagnostic markers for resistance to *D. noxia* remain elusive. Factors that may have impeded critical investigation, thus blurring the accumulation of a coherent body of information applicable to *Dn* resistance, are discussed. This review calls for a more fastidious approach to the interpretation of results, especially considering the growing evidence pointing to the complex regulation of aphid resistance response pathways in plants. Appropriate reflection on prior studies, together with emerging knowledge regarding the complexity and specificity of the *D. noxia*–wheat resistance interaction, should enable scientists to address the challenges of protecting wheat against this pest in future.

**Keywords:** *Diuraphis noxia* (Kurdjumov); host plant resistance; insect-resistance breeding; marker-assisted selection; *Triticum aestivum* L.

1. **Introduction**

The Russian wheat aphid (RWA; *Diuraphis noxia* (Kurdjumov), (Homoptera: Aphididae)) has been known as a severe pest of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) since devastating losses were reported in the Crimea in 1901 [1], as quoted by [2]. This atypical grain aphid now appears throughout the world [3–14], following the 2016 report of its arrival in Australia [15]. Sometimes where *D. noxia* occurs, population levels remain below injurious levels. Damage is however regularly reported in areas characterized by medium-to-lower yield potentials, rain-fed conditions and sporadic droughts [16]. Several traits contribute to causing severe yield loss (60% or more) if this aphid is not controlled [17]. *D. noxia* infestation leads to dramatic chlorotic streaks on leaves [18],
leaf-rolling, general stunting, and head-trapping [19] resulting in a sizeable loss of the photosynthetic area. Furthermore, *D. noxia* has a low developmental-threshold temperature [19], a vast host range throughout the grasses [20], and is protected from many generalist natural enemies by the rolled-leaf pseudo-gall it engenders [21]. Climate change and increased crop pest dispersal make finding tools for breeding resistant cultivars so as to control *D. noxia* more important than ever before.

In South Africa, *D. noxia* control was achieved using resistant cultivars, which formed the basis of an integrated pest management program [22]. Since 1992, >40 *Dn*-resistant (*Dn* after *D. noxia*) wheat cultivars have been released for cultivation. Research, started in 1978, successively focused on four South African *D. noxia* biotypes, namely RWASA1, RWASA2, RWASA3 and RWASA4, with an additional biotype, RWASA5, reported in 2019 [23]. These biotypes now occur concurrently in wheat-producing areas of the country [24–27]. Providentially, numerous sources of *Dn* resistance were rapidly identified following the incursions of *D. noxia* in both South Africa [28] and the United States [29]. On-going research [30–53] provided an ample number of accessions with genetic resistance to this pest. Numerous different mechanisms of resistance to *D. noxia* occur in these sources.

A 2016 review [54] concluded that, for aphid–plant interactions, multiple mechanisms could function at different stages of the interaction, and that these could differ for species pairs at different stages of co-evolution. Furthermore, the stealthy nature of aphid feeding in phloem makes these interactions highly distinct [54]. Within the known sources of *Dn* resistance, large variation occurs with respect to the mechanisms of resistance (antibiosis, antixenosis and tolerance [55]) that are expressed. Different plant metabolic processes of resistance were reviewed by [56]. Considerable evidence points to the role of phloem as a signaling network in addition to its primary role in the partitioning of photo-assimilates [57].

*Dn* resistance, which maintains chlorophyll functionality and thus yield under *D. noxia* infestation, was deployed in winter/facultative cultivars (Supplementary Figure S1 Map of South African wheat production regions) with considerable success and economic benefit [58]. Conventional back-cross-breeding, and the phenotypic screening of host plant resistance using bioassays with live aphids, was used to breed these cultivars [51]. Recently, marker-assisted selection (MAS) for *Dn* resistance breeding has been explored to facilitate gene/quantitative trait loci (QTL) stacking in order to achieve durable resistance to different pests/diseases or several biotypes of the same pest [59–61]. This could replace the phenotypic screening of plants [62,63] with a faster and higher throughput methodology.

Combining R-genes is no guarantee that resistance will be improved or more durable. There are arguments both for and against stacking aphid resistance genes in single accessions. For *Acyrthosiphon kondoi* Shinji (blue alfalfa aphid) resistance in *Medicago truncatula*, gene stacking enhanced aphid resistance with a complex interaction between genes in the pyramid [64,65]. The combination of R-genes *Rag4* and *Rag1b* against *Aphis glycines* (soybean aphid), however, resulted in very susceptible progeny [66]. Importantly, the durability of successful gene stacks is not yet predictable. Naturally occurring R-gene groups named ‘hot spots’ occur where genes that confer resistance to aphids, other insects and pathogens occur together [67]. Some aphid R-genes/QTL identified to date appear to be pleiotropic [68,69], and others epistatic [65,70]. Much research is needed to fully understand how R-gene combinations function. An alternate option to stacking R-genes is the use of mosaic-planting of crop cultivars with different R-genes. This production practice challenges pests with a complex genetic environment, which has been shown to decrease pest fitness [71–73].

1.1. Chronicling Marker Development for *D. noxia* Resistance

1.1.1. Initial Studies

The search for *Dn* resistance markers in bread wheat began in South Africa and the USA in the early 1990s, using RAPD, RAPD-SCAR, PCR-RFLP and RFLP markers to explore donor landraces and near-isogenic lines [74–78]. As new marker technology developed, it was harnessed. By 2001, microsatellite/simple-sequence repeat (SSR) markers on chromosome 7D had been identified to
tag \textit{Dn resistance} genes. The gene \textit{Dn}2 was sub-divided into three “types” based on band size heterogeneity [79], while SSRs with specific size bands were reported to mark \textit{Dn}1, \textit{Dn}2, \textit{Dn}5(sic), \textit{Dnx}, \textit{Dn}8 and \textit{Dn}9 [80]. Ambiguity ensued regarding the location and naming of \textit{Dn}5 following this paper [80,81]. New SSR markers on chromosome 1D for \textit{Dn}4 and \textit{Dn}6 [60] followed shortly, while a \textit{Dn}1 marker was confirmed [82], as cited by [83]. \textit{Dn}4 markers \textit{Xgwm}106 and \textit{Xgwm337}, with estimated genetic distances of 7.4 and 12.9 cM [60], were confirmed in a second study with shorter linkage distances (5.9 and 9.2 cM, respectively) and slightly different band sizes using a different \textit{F}_{2.3} population [84,85]. As with the 7D marker studies, variance between 1D marker studies caused confusion.

In 2005, the authors of [86] attempted to clarify the inconsistency in literature regarding the location and genetic relationships of the \textit{Dn resistance} genes on 7D, namely \textit{Dn}1, \textit{Dn}2, \textit{Dn}5, \textit{Dn}6 and \textit{Dnx}. This study also included five additional donor accessions with uncharacterized \textit{Dn}-genes. It concluded that the majority of \textit{Dn}-genes on 7D are located on the 7DS arm, and that the genes appear either allelic or are tightly linked to one another in a \textit{Dn}-gene cluster. A smaller resistance cluster was confirmed on chromosome 1DS [86] with \textit{Dn}4 [60] forming a part of this cluster. The position of \textit{Dn}5, however, remained contested.

Monotelosomic 7DL plants carrying \textit{Dn}5 on the telosome were developed, and both the 7DS and 7DL telosomes were confirmed using mapped microsatellite and endopeptidase markers to show unequivocally that \textit{Dn}5 occurs on 7DL [87]. This 2006 study found an unknown \textit{Dn}-gene, derived from the same donor as \textit{Dn}5, i.e., PI 294994, on 7DS, substantiating the findings of a cluster on 7DS [86]. This \textit{Dn resistance} gene on 7DS [80,87] has remained unnamed and is referred to in this paper as \textit{DnUnknown}.

1.1.2. Diverse Approaches to \textit{Dn} resistance Marker Identification

Argentinian studies from 1999 onward focused on the identification and mapping of antibiosis and antixenosis to \textit{D. noxia} [68,88]. A 2004 study [69] reported markers \textit{Xpsr687} on 7DS and \textit{Xgwm437} on 7DL for antixenosis, \textit{Xpsr}490 and \textit{Rc3} on 7DS, and \textit{Xgwm44}, \textit{Xgwm}437 and \textit{Xgwm}121 on 7DL for antibiosis, with at least two QTL in the repulsion phase, one near the centromere (7DS or 7DL) and the other distal on 7DL for antibiosis. In 2005, loci \textit{Xgwm}1293 and \textit{Xgwm}1150 on 6AL were associated with antixenosis against a new biotype present in Argentina [89].

By 2007, the research focus for \textit{Dn resistance} markers shifted to genes effective against multiple \textit{D. noxia} biotypes. Resistance breaking biotypes had, by that time, occurred in both the USA [90] and South Africa [25]. Markers were developed for the \textit{Dn resistance} genes \textit{Dn}7 [91] and \textit{Dn}2414 [92]. However, both genes are associated with the “sticky dough” trait from the donor 1RS:1BL wheat-rye translocation. This regrettably made them unsuitable for use in bread wheat breeding programs.

Efforts from 2010 thus focused on the bread wheat accession, CIttr 2401 (PI 9781), as it is also resistant to multiple \textit{D. noxia} biotypes. A study [93] of a doubled haploid population identified numerous QTL associated with the foliar area (\textit{Xpsp}3103 on 4DS, and \textit{Xgdm}3 on 5DS), chlorophyll content (\textit{Xgwm}533 on 3BS and \textit{Xpsp3094} on 7AL) and number of expanded leaves (\textit{Xwmc}264 on 3AS and \textit{XwPt}8836 on 4DS). Pleiotropic effects between the 4DS QTL and \textit{Rht-D1} were noted, as were associations with orthologs of the markers [93]. Further scrutiny of CIttr 2401 saw three papers [94–96] published, documenting the genetic basis of the \textit{Dn}2401 resistance gene, which was mapped to 7DS. Four SSR markers, \textit{Xcf}d68, \textit{Xbarc}214, \textit{Xgwm}473 [94,96] and \textit{Xcf}d14 [96], and two single nucleotide polymorphisms (SNP), \textit{Xowm}705 and \textit{Xowm}711 [96], were identified closer to the \textit{Dn}2401 gene region through focused genetic studies. A 2019 \textit{Dn}2401 study [97] identified new SNP markers (\textit{Xowm}713, \textit{Xowm}714, \textit{Xowm}715 and \textit{Xowm}717) to delineate a 0.3 cM and 133.2 kb interval which contains six high-confidence resistance gene candidiates. Again, several credible studies have stimulated new questions.

Genome-wide association studies (GWAS) were also conducted to identify loci/chromosome regions that control \textit{Dn resistance}. In 2013, an ICARDA study using 134 diverse wheat accessions [98] identified marker \textit{wPt}-733729 (7DS) associated with the leaf curling caused by \textit{D. noxia}, as well as three
markers, namely \textit{wPt-3018} (7DL), \textit{wPt-3291} (7DL) and \textit{wPt-665471} (7DS), associated with leaf chlorosis. In 2016, Australian research identified new QTL for \textit{Dn} resistance that mapped to chromosome 7DS [99]. This study hypothesized that the active area on 7DS, close to the centromere, is controlled by several loci, each providing small additive effects. These loci are tightly linked, segregate together, and may be a single locus comprising multiple alleles associated with specific phenotypes. A novel model was proposed suggesting that the \textit{Dn}-genes at the 7DS locus are possibly contained within a chromatin loop [99].

Sadly, the markers reported above have not been properly validated in multiple wheat backgrounds, and the questions raised regarding pleiotropic effects and marker orthologs were never answered. To illustrate the enigmatic literature, five well-studied SSR markers are listed together with the reported band size for each linked \textit{Dn}-gene/QTL (Table 1). The applicable \textit{Dn} resistance donor accession used in each study, or the accession from which the study material was developed, is provided with the reference to the relevant study. It is prudent to note that all the \textit{Dn}-genes mentioned in Table 1 (\textit{Dn1}, \textit{Dn2}, \textit{Dn5}, \textit{Dn6}, \textit{Dn8}, \textit{Dnx}, \textit{DnUnknown}, \textit{Dn2401} and \textit{Dn626580}) are considered to occur on chromosome 7D near the centromere, but their exact position and how they interact with each other (i.e., alleles or part of an R-gene cluster) is still not yet entirely clear [49,60,80,86,87,95].

| Marker | Fragment Size (bp)/QTL Additive Effect | \textit{D. noxia} R-Gene | Donor/Test Accession(s) | Reference |
|--------|----------------------------------------|--------------------------|--------------------------|-----------|
| \textit{Xgwm44} | Four fragments between 80–182 None (Chinese Spring, Thatcher) # | | [60,86] |
| \textit{Xgwm44} | 185 None (Chinese Spring) | \textit{DnUnknown} | PI 294994 | [87] |
| \textit{Xgwm44} | 180 \textit{Dn6} | PI 243781 | [60] |
| \textit{Xgwm44} | 180 \textit{Dn6} | PI 262660(sic) | [60] |
| \textit{Xgwm44} | 200 \textit{Dn6} | PI 047545 | [60] |
| \textit{Xgwm44} | Instar duration −0.797 ** | QTL \textit{DnUnknown} | Doubled-Haploid Recombinant population of CS and 7D Synthetic | [69] |
| \textit{Xgwm111} | Three fragments between 130–305 None (Chinese Spring, Thatcher) # | | [60] |
| \textit{Xgwm111} | 209 None (Chinese Spring) | | [87] |
| \textit{Xgwm111} | 200 \textit{Dn2} | PI 262660 | [80] |
| \textit{Xgwm111} | 200 \textit{Dn6} | PI 243781 | [60] |
| \textit{Xgwm111} | 210 \textit{Dn1} | PI 137739 | [87] |
| \textit{Xgwm111} | 210 Not yet named | PI 047545 | [60] |
| \textit{Xgwm111} | 215 \textit{DnUnknown} | PI 294994 | [87] |
| \textit{Xgwm111} | 220 \textit{Dn5} | PI 294994 | [87] |
| \textit{Xgwm111} | 225 \textit{Dnx} | PI 220127 | [86] |
| \textit{Xgwm111} | 274 \textit{Dn2401} | CIt 2401 | [95] |
| \textit{Xgwm111} | 210, 240, 250 \textit{Dn1} | PI 137739 | [83] |
| \textit{Xgwm111} | 210, 240, 250 \textit{Dn5} | PI 294994 | [83] |
| \textit{Xgwm111} | 210, 240, 250 None (Chinese Spring 7DS dt) | | [83] |
| \textit{Xgwm437} | 112 None | \textit{Dn2401} | PI 626580 | [49] |
| \textit{Xgwm437} | 100 (Type III) \textit{Dn2} | PI 262660 | [79] |
| \textit{Xgwm437} | 102 (Type II) \textit{Dn2} | PI 262660 | [79] |
| \textit{Xgwm437} | 104 (Type I) \textit{Dn2} | PI 262660 | [79] |
| \textit{Xgwm437} | 105 \textit{Dn5} | PI 294994 | [87] |
| \textit{Xgwm437} | 124 \textit{Dn626580} | PI 626580 | [49] |
| \textit{Xgwm437} | Antixenosis +2.077 ** \textit{Dn2401} | QTL Doubled-Haploid Recombinant population of CS and 7D Synthetic | [69] |
| \textit{Xgwm473} | 244 \textit{Dn626580} | PI 626580 | [49] |
| \textit{Xgwm473} | 244 \textit{Dn2401} | CIt 2401 | [95] |
| \textit{Xgwm635} | 100 \textit{Dn8} | PI 294994 | [87] |

# \textit{Xgwm44182} and \textit{Xgwm111205} are considered characteristic or functional fragments. See [60] for discussion. **, ***: Significant at \( p = 0.01 \) and \( p = 0.001 \), respectively.
Other credible additional factors can be deduced from the literature in hindsight, and may shed light on significant aspects that could inadvertently have influenced this research field. The primary aim of this paper is thus to discuss the sometimes-contradictory literature pertaining to Dn resistance markers on chromosome 7D of wheat, and suggest plausible interpretations of the collective body of literature. Additional examples, obtained by testing some published SSR markers associated with Dn resistance on individual plants with known phenotypes from a panel of South African wheat accessions, will be presented. Prospective avenues for future research are alluded to, considering exciting current developments in the understanding of the complexities of the aphid–host plant resistance interaction.

2. Results

The mean phenotypic damage rating for the five example plants from each accession was used to rank them, from most resistant to least resistant to biotype RWASA2, and calculate the standard error of means, which is presented in Table 2 together with postulated potential genes in the accession.

Table 2. Rank of test entries using the t-distribution test (p = 0.05) of the mean damage rating (SEM) of each accession to biotype RWASA2.

| Accessions Ranked from Most Resistant to Least Resistant | Mean RWASA2 Damage Rating (SEM) of Five Individual Example Plants of Each Accession | Postulated Potential Gene(s) in the Accession |
|---------------------------------------------------------|---------------------------------------------------------------------------------|---------------------------------------------|
| PI 137739’S’ | 3.0 (0) | Dn1 |
| CIt 2401 | 3.2 (0.5) | Dn2401 |
| T06/16 | 3.2 (0.4) | Dn1, Dn5, Dn8, Dn9, DnUnknown |
| PI 586954 | 3.4 (0.5) | Dnx |
| PI 47545 | 3.8 (0.4) | Dn7545 |
| PAN 3144 | 4.0 (0) | Gene not known |
| PI 626580 | 5.0 (1.1) | Dn626580 |
| PI 586955 | 5.2 (1.9) | Dn4 |
| T06/13 | 5.8 (2.7) | Dn5, Dn8, Dn9, DnUnknown |
| PI 243781 | 6.2 (2.6) | Dn6 |
| PI 294994 | 6.8 (2.3) | Dn5, Dn8, Dn9, DnUnknown |
| T03/17 | 7.6 (2.2) | Dn1, Dn2 |
| T05/02 | 7.8 (0.4) | Dn5, Dn8, Dn9, DnUnknown |
| PI 262660 | 8.0 (0.6) | Dn2 |
| TugelaDn2 | 8.2 (0.4) | Dn2 |
| Yumar | 8.2 (0.7) | Dn4 |
| BW991306 | 8.4 (0.8) | Dn2401, Dn5, Dn8, Dn9, DnUnknown |
| BW991405 | 8.4 (0.5) | Dn2401, Dn5, Dn8, Dn9, DnUnknown |
| PI 634775 | 8.5 (0.9) | Dn8 |
| RIL-A50 | 8.6 (0.5) | Dn2401 |
| Tugela-DN | 8.8 (0.4) | Dn1 |
| Betta-DN | 9.0 (0) | Dn1 |
| Gariep | 9.0 (0) | Dn1 |
| BetaDn2 | 9.0 (0) | Dn2 |
| Hugenoot | 9.0 (0) | Susceptible control |
| PI 634770 | 9.2 (0.4) | Dn9 |

2.1. Phenotyping

RWASA2, first reported as “Clone 2” [25], is virulent to Dn1, Dn2, d3, Dn8 and Dn9 [27], while the genes Dn4, Dn5, Dn6, Dn7, Dnx and Dny remain effective against this biotype [27]. When considering the pedigrees of the accessions in the panel (see M&M), it is expected that several of them should be susceptible to RWASA2. This includes the susceptible control Hugenoot, as well as PI 137739’S’ (Dn1), Betta-DN (Dn1), Gariep (Dn1), Tugela-DN (Dn1), PI 262660 (Dn2), BetaDn2, TugelaDn2, PI 634775 (Dn8), PI 634770 (Dn9) and T03/17 (Dn1, Dn2). The data confirm that, with the exception of PI 137739’S’, all the accessions one would expect to be susceptible to RWASA2 are indeed susceptible.
There are, however, a number of accessions that are postulated to contain *D. noxia* resistance genes that should confer resistance to RWASA2, which are susceptible. It could thus be construed that T05/02 does not contain *Dn5*, Yumar does not contain *Dn4*, neither breeding-lines BW991306 nor BW991405 contain *Dn2401* or *Dn5*, and RIL-A50 does not contain *Dn2401*. PI 137739"S", a selection from the original landrace PI 137739, must then contain either an additional gene to the reported *Dn1*, or a different gene that confers RWASA2 resistance. In addition to PI 137739"S" (*Dn137739"S"*), accessions CIt 2401 (*Dn2401*), T06/16 (*Dn5, Dn8, Dn9, DnUnknown*), PI 58654 (*Dnx*), PI 47545 (*Dn47545*), PAN 3144 (gene not known) and PI 626580 (*Dn626580*) tested as being resistant to RWASA2, with PI 586955 (*Dnx*), T06/13 (*Dn5, Dn8, Dn9, DnUnknown*), PI 243781 (*Dn6*) and PI 294,994 (*Dn5, Dn8, Dn9, DnUnknown*) testing as being moderately resistant to this biotype.

Notably, multiple *D. noxia* biotypes [27,100] occur concurrently in wheat fields in South Africa, although the predominant biotype may vary from season to season and within particular geographic regions. This requires that genes with resistance to different biotypes be combined within a single accession order to make multiple-biotype resistant cultivars available to producers. Due to the variation in resistance reactions present in different plants of the same accession, it is not possible to stack *Dn* resistance against different biotypes without diagnostic molecular markers. A single plant can only be accurately phenotyped with one biotype in each generation. For example, a robust molecular marker for any gene(s) resistant to RWASA1 but susceptible to RWASA2 would enable breeders to combine the RWASA1-effective gene(s) with RWASA2-effective gene(s), for the control of more than one biotype concurrently. Screening the germplasm with RWASA2 would identify plants with RWASA2-effective resistance, and RWASA1-effective resistance could be identified by selecting those RWASA2-resistant plants that also contain the marker. The reciprocate is not possible, as most genes effective against RWASA2 would mask the presence of genes effective against RWASA1 (Personal communication, data not shown VL Tolmay) if the plants were phenotyped with the RWASA1 biotype. This seeming anomaly could easily be explained if the particular *Dn resistance* in these accessions is complex in nature, and is contingent on the *D. noxia* biotype used to develop/select the accession, with other biotypes either recognizing the whole or only parts of the complex resistance.

2.2. Genotyping

Of the five markers tested on this panel of accessions, *Xgwm473* and *Xgwm635* did not reflect sufficient polymorphism, and the data are therefore not shown. *Xgwm473* was reported to be linked to *Dn* resistance by two studies [49,95], with both groups describing a 244 bp fragment as the diagnostic band. However, the genes reportedly linked to this fragment were different, namely, *Dn626580* [49] and *Dn2401* [95]. *Xgwm635* was reported to be linked to *Dn8* from PI 294994 [80] with a 100 bp band. The three remaining markers, namely *Xgwm44*, *Xgwm111* and *Xgwm437*, for which PIC values [101] were calculated from the panel data (Supplementary Table S1), will be discussed below in the order they occur on the wheat consensus map [102] of chromosome 7D. It is, however, prudent to note that markers *Xgwm44* and *Xgwm111* have multiple orthologs, as reported [86,103] (Supplementary Table S1), potentially compounding allelic interpretations.

SSR marker *Xgwm44*, located on 7DS [80,86,104], is reported to give a 180 bp band for resistance gene *Dn6* from accessions PI 243781 [60,86] and PI 262660 (sic) [86], while resistance from accession PI 047545 was linked to a 200 bp fragment [86]. A 180 bp fragment was also reported for this marker for *DnUnknown* [87]. In this study, 12 haplotype combinations (Supplementary Table S1) of band sizes 0, 120, 130, 150, 175, 185, 190 and 200 bp were found in both individual resistant and susceptible plants.

Wheat microsatellite marker *Xgwm111*, on the short arm of chromosome 7D [86], has been associated with *Dn resistance* since the report [80] that it is tightly linked to *Dn1, Dn2, Dn5*(sic) and *Dnx*. The single band sizes reported for each of the genes in this study [80] were 210 bp [PI 137739], 200 bp [PI 262660], 220 bp [PI 294994] and 225 bp [PI220127], respectively. The resistance gene *Dn5*(sic) from PI 294994 identified by [80] is probably not the same as *Dn5*, named by and allocated to chromosome 7DL through a telosomic analysis [81]. A follow-up study [87] using *Xgwm437* placed
Dn5 on 7DL, as it was only amplified in 7DL monotelosomic plants. This corroborates the prior mapping [102,104] of Xgwm437 on 7DL. Furthermore, the landrace PI 294994 is known to contain several different resistance variants [105,106]. The most widely accepted explanation for the confusion regarding resistance genes from this landrace is that the resistant plants used in these and other studies [80,81,105,106], though all linked to landrace PI 294994, differ from each other because different single plants were selected for use. The biotype used for the phenotypic evaluation of the plant reaction could be an additional factor contributing variability to the results, as the contradictory genetic studies identifying Dn5 used different D. noxia biotypes, namely RWASA1 [81,87] and RWA1 [80], to evaluate for susceptible and resistant plants. Furthermore, the close proximity of the D. noxia R-genes to the centromere of chromosome 7D significantly affects recombination frequencies and further hinders clarity [87]. The literature reports Xgwm111 band sizes 200, 210, 215, 220, 225 and 274 bp associated with the phenotypic expression of Dn resistance (Table 1). In this study, more allelic variation was observed with band sizes 0, 130, 135, 150, 180, 190, 200, 210 and 220 bp recorded in 16 haplotype combinations (Supplementary Table S1) from both resistant and susceptible plants. None of the plants in this study gave a band size of 215 bp [87], 225 bp [80] or 274 bp [95], despite the donor accessions PI 294994 and C1tr 2401, in addition to numerous accessions developed from these accessions, present in the test panel.

Three different ‘types’ of bands were found with marker Xgwm437, located on 7DL [79], that were associated with resistant plants derived from accession PI 262660 (Table 1). These fragments were reported as the ‘highest bands’, and the illustration provided in this manuscript clearly shows multiple bands obtained with this marker. These three ‘highest bands’ with band sizes 104 bp (Type I), 102 bp (Type II) and 100 bp (Type III) are very close in size to the 105 bp band reported for Dn5 [87] from PI 294994. A 105 bp fragment was also reported to be associated with Dn626580 [49]. In this study, each of the 11 haplotypes (Supplementary Table S1) contained a single band of either 0, 90, 95, 100, 105, 110, 115, 120, 125, 130 or 135 bp for this panel. These haplotypes appear to occur in specific combinations with the haplotypes associated with Xgwm111 and Xgwm44, alluding to the existence of a diverse resistance cluster or a block of allelic variants.

2.3. Correlation of Phenotype and Marker Results

To practically illustrate selection, using a combination of the phenotype resistance expression of one D. noxia biotype (in this case, RWASA2) and SSR markers for resistance to another biotype (for arguments sake, RWASA1), let us consider some examples (Table 3). Accessions derived from single R-gene-sources will be briefly discussed, before moving to those with potential combinations of R-genes from multiple sources.

### Table 3. Accession (Sample name), D. noxia damage score (RWASA2) and marker haplotype for single plant examples screened with markers Xgwm44, Xgwm111 and Xgwm437.

| Accession (Sample Name) | Single Example Plant RWASA2 Score | Xgwm44 | Xgwm111 | Xgwm437 |
|-------------------------|-----------------------------------|--------|---------|---------|
| Betta-DN_1              | 9                                 | 120; 190 | 135; 210 | 120     |
| Betta-DN_2              | 9                                 | 120; 190 | 135; 210 | 120     |
| Betta-DN_3              | 9                                 | 120; 190 | 135; 210 | 120     |
| Betta-DN_4              | 9                                 | 120; 190 | 135; 210 | 120     |
| Betta-DN_5              | 9                                 | 120; 200 | 135; 220 | 120     |
| Gariep_1                | 9                                 | 120; 190 | 135; 210 | 115     |
| Gariep_2                | 9                                 | 120; 190 | 135; 210 | 115     |
| Gariep_3                | 9                                 | 120; 190 | 135; 210 | 115     |
| Gariep_4                | 9                                 | 120; 190 | 135; 210 | 115     |
| Tugela-DN (V4483)       | 9                                 | 120; 190 | 135; 210 | 115     |
| Tugela-DN (V4484)       | 9                                 | 120; 190 | 135; 210 | 115     |
| Tugela-DN (V4485)       | 9                                 | 120; 190 | 135; 210 | 115     |
| Tugela-DN (V4486)       | 9                                 | 120; 190 | 135; 210 | 115     |
Commercial cultivars Betta-DN, Gariep and Tugela-DN (all derived from PI 137739 and potentially containing Dn1) tested as being susceptible to RWASA2. They all contained the Xgwm44 and Xgwm111 haplotypes, while Betta-DN contained Xgwm437, and both Gariep and Tugela-DN contained Xgwm437
115
(Table 3). Advanced breeding-lines BettaDn2 and TugelaDn2 (derived from PI 262660 and potentially containing Dn2) were also susceptible to RWASA2. Within the 10 plants representing these two advanced breeding-lines, 9 plants contained the Xgwm44 haplotype with 1 TugelaDn2 plant containing Xgwm44 null. All five BettaDn2 plants as well as two of the TugelaDn2 plants contained haplotype Xgwm111
135;210
as well as Xgwm437
100
. The remaining three TugelaDn2 plants contained Xgwm111
135;220
and Xgwm437
115
. It is not unequivocally possible to confirm the presence of Dn1 or Dn2 based on these marker alleles, although the phenotypic data using RWASA2 is expected for plants containing these genes.

Both T05/02 plants and two of the T16/03 plants (derived from PI 294994 using RWASA1) tested as being susceptible to RWASA2, suggesting that they do not contain Dn5, although they may well contain Dn8, Dn9 and DnUnknown, or any combination of the latter. The remaining two plants of T06/13 were resistant (a damage rating score of 6 or less) to RWASA2, and contained the Xgwm44
120;175
, Xgwm111
135;200
and Xgwm437
120
haplotypes. The susceptible plants contained different haplotypes, namely Xgwm44
null
, Xgwm111
135;210
and Xgwm437
100
 (1 T05/03 plant); Xgwm44
120;175
, Xgwm111
135;200
 and Xgwm437
100
 (1 T05/03 plant) or Xgwm44
120;185
, Xgwm111
135;200
 and Xgwm437
120
 (2 T06/03 plants).

Again, based on the above data, it is not possible to definitely confirm Dn5 present in the two RWASA2-resistant plants. These individual plants cannot be rescreened using RWASA1 or any other
biotype, and there is no guarantee that their progeny or other seeds from the same mother plant will have the same haplotypes as these individual plants.

Advanced breeding-lines T03/17 (Dn1 and Dn2) and T06/16 (Dn1 and Dn5, Dn8, Dn9, DnUnknown) were purposefully developed to combine Dn-genes from multiple sources. The haplotypes of the five T03/17 plants are Xgwm44120;175, Xgwm111130;200 and Xgwm437120, and all are susceptible to RWASA2. Similar to the reasoning for accessions containing either Dn1 or Dn2, these results do not confirm the presence of either genes, nor whether the attempt to combine them was successful. According to the pedigree, advanced breeding-line T06/16 could potentially contain Dn1, Dn5, Dn8, Dn9 and DnUnknown, or any combination of these genes. All five plants of this accession tested as being resistant when screened with RWASA2, phenotypically substantiating the postulated presence of Dn5 as the only one of these genes reported to confer resistance to RWASA2. Haplotypes Xgwm44120;175, Xgwm111130;200 and Xgwm437135 occur in four plants with a higher level of resistance than the fifth, which contains marker haplotype Xgwm44120;190 instead of the 120; 175 bp band recorded for the other single plants. Again, the marker haplotypes do not correspond with the published information for Dn5 or DnUnknown, and it is not clear whether Dn1 is present in these plants at all. Had these plants been screened with RWASA1, the presence of Dn5 (which was substantiated in this case by the RWASA2 screening that took place) would have masked the presence of Dn1, as both genes confer resistance to RWASA1.

The phenotypic reaction of resistant commercial cultivar and the check accession of PAN 3144 (see M&M) shows that it contains gene(s) conferring resistance to RWASA1, RWASA2 and RWASA3. Biotype characterization studies [27,100] list Dn5, Dn6, Dn7 and Dn8 as the only genes that confer resistance to all three of these biotypes. The haplotypes of the five resistant plants, namely Xgwm44120;190 or 195, Xgwm111135;200 or 210 and Xgwm437120, do not clearly indicate the presence of any of those genes in these plants.

Single plant data pertaining to the landrace R-donors and other accessions included in this study can be found in Supplementary Table S1. The five plants of landrace PI 137739"S" are uniform in terms of their genotype, as are those of PI 262660. In all likelihood, this is due to a specific, targeted selection in the case of PI 137739"S", where a single plant with resistance to biotype RWASA3 = 5 was selected in January 2015. PI 262660 may inadvertently have become more uniform over many years of successive use and seed multiplication. Three haplotypes are present in the five plants of PI 294994, with only two plants, of the same haplotype, testing resistant to RWASA2. All four plants of landrace PI 047545 tested resistant to RWASA2, with the most resistant plant possessing a different haplotype to the others. Two haplotypes were also contained in the landrace PI 243781, with the single resistant plant different to the other susceptible ones, while in PI 626580 two haplotypes are present, but the most susceptible plant has the same haplotype as one of the most resistant. All three plants of CItr 2401 were resistant and contained the same haplotype. It would appear that the allelic diversity in landrace accessions may be dependent on whether the accession is still an amalgamation (bulked-up as collected) or whether a selection has been purified from it. Furthermore, allelic variation may be dependent on, or restricted by, which D. noxia biotype was used to characterize the accession or make the selection. The potential influence of the biotype used during the screening and selection would furthermore naturally affect/influence the robustness of the marker allele’s association with the trait. Generally, the haplotype data for the individual plants of single R-gene cultivars and advanced breeding-line accessions are sufficiently uniform to indicate that the accessions are true breeding. In the case of accessions developed to combine genes, the variation is somewhat greater.

3. Discussion

Many authors make an important distinction between markers useful for MAS and those that are not [103,107–109]. Generally, three critical requirements distinguish markers considered functional or diagnostic for MAS [107,110]. These are reliability, repeatability and robustness. The first requires flanking markers or tight (≤1 cm) linkage between the marker and the target gene/trait [108], as a larger
distance can result in false selection [76,108]. The second is the validation of the marker–trait association across multiple genetic backgrounds [108,111], and the third is the suitability for large scale commercial application [108,109] versus the gain per unit time and cost [103]. The examples presented in this paper indicate that the markers tested did not meet the required level of reliability and repeatability across a panel of resistant and susceptible South African accessions. This is possibly due to the relatively large linkage distances and a lack of conclusive validation studies. In general, these examples show that multiple haplotypes exist in many of the test accessions, even when the phenotype is similar, and the accession is of an advanced enough generation to expect a true breeding response.

Two inter-related sources of ambiguity can be identified, which could account for the observed phenomena. Firstly, in terms of the host plant genetics, the dominant inheritance of most Dn resistance, often attributed to single genes [32,38,41,42,47–49,52,56,81,105,106,112–115], has underpinned many studies, despite evidence of a more complex genetic control of resistance [16,40,53,60,68,69,80,86,89,98,99,116]. ‘Downstream’ support for the last-mentioned hypothesis includes studies reporting multiple resistance mechanisms present in specific accessions [40,117–119], QTL associated with specific mechanisms [68,69,88,89,93,99], and studies reporting differential gene expression [120–123]. Additional compelling results include a 2009 paper [118] reporting multiple loci of genetic control within a single accession. Breeding-line KS94H871, containing Dnx from PI 220127, was shown to contain two loci encoding resistance to RWA1, but only one locus encoding resistance to RWA2. This pattern is echoed in the 2016 GWAS of a DH population (ECA Gregory × PI 94365) from Australia [99], with two loci (on 7D and 1D) encoding resistance to RWASA1 and RWASA2, while only the 1D locus encodes resistance to RWASA3. The re-evaluation and selection of resistant plants from ‘Plant Introduction’ accessions following the discovery of biotype USA2 in the United States [53] could point to mixed landrace accessions, as stated by the authors, but could alternately be explained by the “Dn-biotype-specific–R-gene(s)” concept shown in the aforementioned studies [99,118].

The second source of confusion could result from the D. noxia biotype used for the phenotypic evaluation of wheat accessions used in specific studies. This is true for the evaluation during the development of the test population and/or the evaluation of the phenotype which is used for trait-association analysis. In some instances, the biotype(s) used for the initial development of study-accessions and association mapping studies are not the same, while in some they are. The biotype is rarely specified per “Dn-marker–R-gene association”. Furthermore, Dn markers have rarely been validated using different/multiple biotypes to assess the specificity of the marker–trait association. Table 4 contains a summary of germplasms utilized in 7D marker studies, listing the D. noxia biotype(s) used to develop the accessions and evaluate the phenotype for genotypic association/marker development.

Table 4. Biotype(s) used for selection and/or development of wheat accessions, for the linkage analysis phenotyping and 7D marker alleles reported in the literature.

| Accession(s) Selected or Developed with D. noxia Biotype | D. noxia Biotype Used to Phenotype for Linkage Analysis | 7D Markers Found (Reference) |
|----------------------------------------------------------|----------------------------------------------------------|-------------------------------|
| PI 137739, PI 262660, PI 294994 selected with RWASA1 * and Betta-Dn1, Betta-Dn2, Betta-Dn9, Tugela-Dn1, Tugela-Dn2, Karee-Dn2, Karee-Dn8 developed with RWASA1 * [124] | RWA1 * | Xgwm111 200, 210, 225 [80] Xgwm635 100 [80] |
| Sando selection 4040 × PI 220127 F2:3 developed with RWA1 [80] | RWA1 | Xgwm111 225 [86] |
| Carson × PI 262660 F2:3 developed with RWA1 [79] | RWA1 | Xgwm437 100, 102, 104 [79] |
| PI 372129, PI 243781, Thunderbird × PI 372129 (Dn4), Wichita × PI 372129 (Dn4), Wichita × PI 243781 (Dn6), and AL359 × PI 243781 (Dn6) developed with RWA1 [60] | RWA1 | Xgwm444 180 [60] Xgwm111 200 [60] |
Table 4. Cont.

| Accession(s) Selected or Developed with D. noxia Biotype | D. noxia Biotype Used to Phenotype for Linkage Analysis | 7D Markers Found (Reference) |
|---------------------------------------------------------|----------------------------------------------------|-----------------------------|
| F2 Beta-Dn1 /Tugela-Dn2 †                              | RWA1                                               | Xgwm444180, 200 [86]         |
| Tugela-Dn1 †                                            |                                                    | Xgwm111216 [86]              |
| F2 Beta-Dn5 /Tugela-Dn1 †                              |                                                    |                             |
| F2 Kario-Dn5 /Tugela-Dn2 †                             |                                                    |                             |
| F2 PI 220127 (Dnx)/Tugela-Dn1 †                       |                                                    |                             |
| F2 PI 220127 (Dnx)/Tugela-Dn2 †                       |                                                    |                             |
| F2 PI 243781 (Dn6)/PI 137739 (Dn1) #                   |                                                    |                             |
| F2 PI 243781 (Dn6)/PI 372129(Dn4) #                    |                                                    |                             |
| TC1 F1 Wichita/(Beta-Dn1 /Tugela-Dn1 †) #             |                                                    |                             |
| TC1 F1 Wichita/(Beta-Dn1 /Tugela-Dn2 †) #             |                                                    |                             |
| TC1 F1 Wichita/(Beta-Dn5 /Tugela-Dn2 †) #             |                                                    |                             |
| TC1 F1 Wichita/(PI 243781 Dn6/PI 137739 Dn1) #         |                                                    |                             |
| NIL 92RL28, (PI 294994/5 ‘Palmiet’) developed with RWASA1 * |                                                    |                             |
| PF626580 × Yuma F2.3 developed with RWA2 [49]          | RWA2                                               | Xgwm437224 [49]              |
| ‘Glupro’ × Cln12401 F2.3 and Cln21041 × ‘Glupro’ F2.3 developed with RWA2 [95]| RWA2                                               | Xgwm473244 [95]              |
| Tugela–Dn2, Tugela–Dn5, Palmiet–Dn5, PI 137739 (=SA1684), PI 262660 (=SA199), PI 294,994 (=SA463), Chinese Spring 7DS dt, Chinese Spring 7DL dt, Tugela, Tugela × Tugela-Dn1 F3, developed with RWASA1 [83]| RWASA1                                               | Xgwm111210, 210, 240, 250 [83] |
| 134 diverse wheat accessions selected with RWASY [98] | RWASY                                               | wPt-733729 [98]              |
|                                                        |                                                    | wPt-665471 [98]              |
|                                                        |                                                    | wPt-3018 [98]                |
|                                                        |                                                    | wPt-3291 [98]                |
| DH mapping population derived from EGA Gregory × PI94365 developed without phenotyping [99]| RWASA1                                               | § QTL_RWASA1_7D [99]         |
|                                                        | RWASA2                                              | QTL_RWASA2_7D [99]           |
|                                                        | RWASA3                                              | QTL_RWATR_rolling_7D [99]    |
|                                                        | RWATR                                               | QTL_RWASA1_7D [99]           |
|                                                        |                                                     | QTL_RWASA2_7D [99]           |
|                                                        |                                                     | QTL_RWATR_rolling_7D [99]    |

T RWASA1 = Original South African biotype; RWASA2 = second South African Biotype; RWASA3 = third South African biotype; RWA1 = Original USA biotype; RWA2 = second USA biotype; RWASY = Original Syrian biotype; RWATR = Original Turkish biotype. * Initial identification and development of near-isogenic-lines with RWASA1 [124], further development with RWA1 [80,125]. † developed with RWA1. Inferred as original USA D. noxia biotype based on year of study. § Inferred as original South African D. noxia biotype based on year of study. § Author original designation modified as follows to reflect common RWA biotype nomenclature: ‘QTL_RWA SAB1_7D’ presented as ‘QTL_RWASA1_7D’; ‘QTL_RWA SAB2_7D’ presented as ‘QTL_RWASA2_7D’; ‘QTL_RWA Trolling_7D’ presented as ‘QTL_RWATR_rolling_7D’.

Inconsistencies with respect to fragment size between different studies, over many years in many wheat accessions, indicate that the marker alleles are not diagnostic. This may be potential Dn-gene allelic variation that has gone unresolved or un-noticed in the past. Nevertheless, the same markers are repeatedly found to be linked to Dn resistance on chromosome 7D. This indicates that genetic resistance to this pest is coded within those regions in some way. The current shortage of diagnostic markers for this trait should be addressed, taking account of the growing evidence for the complex regulation of resistance gene expression [126,127].

Across multiple crops, the complexity of aphid–plant interactions is being progressively revealed [71]. Despite multiple D. noxia biotype studies [128–134], many unknowns still have to be clarified. In general, “research shows that aphid virulence may be a complex adaptation involving a myriad of factors, including epigenetically controlled phenotypic plasticity and contributions from endosymbionts, the gut and saliva” [126]. Likewise, studies of plant defence against insects reveal that resistance gene expression and defence metabolism is influenced by both exogenous and endogenous environmental factors [71].
New evidence shows that plants utilize sophisticated mechanisms to modulate their response to stressors [135]. Embracing these unknowns within the current knowledge base [136], and engaging with them by using the ever-improving understanding of plant defence against insects, may lead to what has eluded us thus far. It is imperative that breeders are enabled with diagnostic markers with which to address the challenges posed by not only the insect pests, but also the changing climatic conditions which will undoubtedly influence pest distribution and the extent of damage they cause. The tools we need to breed D. noxia-resistant wheat will probably be based on a far better understanding of the specific D. noxia–host plant interactions. Two of the current developments in wheat to follow closely involve studies applying advanced molecular technologies to pinpoint D. noxia resistance genes [96,97] and to understand the regulation of the resistance response pathway [127,137,138]. Genetic characterization of the various donor sources of R-gene(s)/QTL, an understanding of the functional plant metabolism encoded by each genetic component, as well as a clear understanding of how these components interact with each other and the specific D. noxia biotype, will be essential to harnessing this plant-resistance to protect wheat in future.

4. Materials and Methods

4.1. Plant Materials

The 26 accession panel (Table 5) used to provide single plant examples in this study is comprised predominantly of wheat cultivars and advanced breeding-lines from the Agricultural Research Council-Small Grain (ARC-SG) D. noxia pre-breeding program, South Africa [139]. Based on pedigree data and phenotypic evaluation with multiple biotypes, the accessions are postulated to potentially contain different Dn-genes (Table 5) or combinations thereof. The wheat cultivars Gariep, Yumar and PAN 3144 are considered differential checks, and their differential RWASA-biotype responses are shown in Table 6 together with those of the susceptible (Hugenoot) and resistant (CItr 2401) controls.

| Wheat Accession | Pedigree | Accession Status | D. noxia R-Gene(s) Potentially Present | Mean (SEM) RWASA1 Score * | Mean (SEM) RWASA2 Score * |
|-----------------|----------|-----------------|--------------------------------------|--------------------------|--------------------------|
| Hugenoot        | Betta/Flamink/Amigo | Cultivar, Susceptible check | None | 9.3 (0.45) | 9.0 (0.58) |
| PI 137739’S”S”  | Not applicable | Selection from Dn1 D. noxia R-donor, Landrace ex. Iran | Dn1 and/or Dn137739’S”S” | 5.1 (1.68) | 4.5 (1.94) |
| Betta-DN        | PI 137739/4Betta(4) | Cultivar | Dn1 | 5.5 (1.74) | 8.2 (1.09) |
| Gariep          | PI 137739/4 Molopo(20) | Cultivar, Differential check | Dn1 | 5.3 (0.55) | 8.0 (1.01) |
| Tugela-DN       | Tugela*4/PI 137739 | Cultivar | Dn1 | 5.4 (1.34) | 7.7 (0.98) |
| PI 262660       | Not applicable | D. noxia R-donor, Landrace ex. Azerbaijan | Dn2 | 4.4 (0.54) | 6.7 (2.20) |
| BettaDn2        | Betta*4/PI 262660 | Advanced breeding-line [SYN = PI 634769] | Dn2 | 5.3 (1.07) | - |
| TugelaDn2       | Tugela*4/PI 262660 | Advanced breeding-line [SYN = PI 634772] | Dn2 | 6.0 (1.18) | - |
| Yumar           | Yuma/PI-37219/CO-850034/3/YD”S”/4*Yuma | Cultivar, Differential check | Dn4 | 5.9 (1.36) | 7.6 (1.82) |
| PI 294994       | Not applicable | D. noxia R-donor, Landrace ex. Bulgaria | Dn5, Dn6, Dn8, Dn10, Dn12 | 4.0 (0.80) | 4.1 (0.25) |
| T05/02          | PI-294994/*4Molen | Advanced breeding-line | Dn5, Dn8, Dn10, Dn12, Dn15 Unknown | 3.9 (1.21) | 3.9 (0.69) |
| T06/13          | Karree/4/PI-294994/*4Garmany/3/YD’S”/BON/Dove”S” # PANAR ® Proprietary information | Advanced breeding-line | Dn5, Dn8, Dn10, Dn12, Dn15 Unknown | 3.9 (1.74) | 3.7 (0.92) |
| PAN 3144        | Not applicable | Cultivar, Differential check | Gene not known | 4.1 (0.80) | 3.5 (0.59) |
| PI 243781       | Not applicable | D. noxia R-donor, Landrace ex. Iran | Dn6 | 3.1 (0.87) | 5.5 (2.02) |
Table 5. Cont.

| Wheat Accession | Pedigree | Accession Status | D. noxia R-Gene(s) Potentially Present | Mean (SEM) RWASA1 Score * | Mean (SEM) RWASA2 Score * |
|-----------------|----------|------------------|---------------------------------------|----------------------------|---------------------------|
| PI 634770       | Karel*6/PI 294994 Advanced breeding-line | Dn8 | 8.1 (1.92) | - |
| PI 634770       | PI 294994/4Betta Advanced breeding-line | Dn9 | 5.6 (0.66) | - |
| PI 566954       | PI 220127/P5/TAM200/KS87H66 Advanced breeding-line | Dnx | 4.4 (0.75) | 4.1 (0.46) |
| PI 566954       | PI 220127/P5/TAM200/KS87H66 Advanced breeding-line | Dnx | 3.2 (1.05) | 4.1 (1.42) |
| PI 047545       | Not applicable | D. noxia R-donor, Landrace ex. Iran | Dn47545 | 3.2 (1.49) | 3.7 (0.74) |
| PI 626580       | Not applicable | D. noxia R-donor, Landrace ex. Iran | Dn626580 | 5.1 (1.35) | 4.5 (1.31) |
| Ctr 2401        | Not applicable | D. noxia R-donor, Landrace ex. Tajikistan | Dn2401 | 3.6 (0.58) | 4.0 (0.58) |
| RIL-A50         | Kavkaz*5/Ctr 2401 Advanced breeding-line | Dn1 + Dn2 | 4.4 (1.11) | 5.1 (1.20) |
| T03/17          | S023331*/P22660//6611–33/ Tugela-DN* (ex. PI 13779) Advanced breeding-line | Dn1 + Dn5, Dn8, Dn9, DnUnknown | 4.1 (1.93) | 3.3 (0.94) |
| T06/16          | Gariepn*6/PI 13779//4/PI 294994//4Gamtoos/3/YD/5°/BON// Dove* | Advanced breeding-line | Dn2401 + Dn5, Dn8, Dn9, DnUnknown | 7.0 (1.49) | 6.4 (1.80) |
| BW991405        | PI 294994/4Molen/Ctr 2401/4Kariega Advanced breeding-line | Dn2401 + Dn5, Dn8, Dn9, DnUnknown | - | 4.9 (2.16) |

* Scores based on visual D. noxia damage to seedlings which is rated from 1 to 10 where 1 = Small isolated chlorotic spots, 2 = Small chlorotic spots, 3 = Chlorotic spots in rows, 4 = Chlorotic splotch, 5 = Mild chlorotic streaks, 6 = Prominent chlorotic streaks, 7 = Severe streaks, leaves fold conduplicate, 8 = Severe streaks, leaves roll convolute, 9 = Severe streaks, leaves roll tightly, and 10 = Plant dying [16]. Means collated from multiple prior evaluations with n ≥ 11 ≤ 40 (Supplementary Table S2). # Note 1: Gamtoos = Veery#3 [140–142] is a susceptible cultivar with the IB/IR translocation released in South Africa in 1983. Multiple resistant accessions were developed from it by ARC-Small Grain Centre, Bethlehem, South Africa, namely Gamtoos-DN (Dn1) [143] GamtoosDn2 and GamtoosDn5 [144] and Stellenbosch University, Stellenbosch, RSA, ‘GamtoosDn7’ [142,143].

RWASA2 was chosen to phenotype the individual plants for marker validation. It is sufficiently damaging to allow discrimination, and all checks (Table 6) give consistent responses to it, while with other resistance breaking biotypes (RWASA3, RWASA4), a measure of segregation is known to occur. The reaction of accessions to the original South African biotype (Supplementary Table S2) was considered the baseline reaction of each accession.

Table 6. Susceptible, differential and resistant checks used in the study, the D. noxia R-genes they reportedly carry and reactions to four South African D. noxia biotypes (Adapted from [145]). A typical damage rating score of 1–3 is considered highly resistant (HR); 4, 5 is resistant (R); 6, 7 is moderately resistant (MR) and 8–10 is susceptible (S).

| Differential Checks | D. noxia R-Gene | RWASA1 | RWASA2 | RWASA3 | RWASA4 |
|---------------------|-----------------|--------|--------|--------|--------|
| Hugnoort            | None            | S      | S      | S      | S      |
| Gariep              | Dn1             | MR     | S      | S      | S      |
| Yumar               | Dn4             | MR     | MR     | S      | S      |
| PAN 3144            | Gene not known  | R      | R      | R      | S      |
| Ctr 2401            | Dn2401          | R      | R      | R      | R      |

4.2. Phenotypic Screening and Tissue Collection from Single Example Plants

A 21-day seedling assay [16] was performed to phenotype the test plants. In total, 15 individual seeds of each accession were planted in Professional Potting Mix® (Cultera, Muldersdrift, South Africa, www.cultera.co.za). Five cones per accession containing three seeds were arranged in a randomized
complete block design within two 98-cone trays and then watered with KynoPop™ (Kynoch, Sandton, South Africa, www.kynoch.co.za) seedling fertilizer. Seven days post-planting, fresh leaf tissue material for DNA extraction purposes was harvested from a single plant per cone for each accession, and the other plants that germinated within that cone were uprooted and discarded. Every accession was left with five individual plants that were then each infested with c. five individuals of apterous mixed instars of *D. noxia* biotype RWASA2. The RWASA2 biotype used in this study was obtained from a colony maintained at ARC-SG. The individual plants were scored 21 days post-infestation using a damage rating scale of 1–10, where 1 = Highly resistant and 10 = Dead [15].

4.3. DNA Isolation and Polymerase Chain Reaction (PCR)

The fresh leaf material, harvested from five individual plants of each test accession, was individually homogenized within 750 µL of extraction buffer for 1 min at 30 r/s with the Qiagen TissueLyser II. A modified cetyltrimethylammonium bromide (CTAB) DNA extraction protocol [146] was used to isolate genomic DNA, which was then treated with 2 µL RNase-A enzyme (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa). A Nanodrop 2000 Spectrophotometer (Thermo Scientific (Pty) Ltd., Waltham, MA, USA) was used to determine the quality, purity and concentration of each sample at the absorbance ratio of 260/280 nm. DNA samples were diluted with 1x TE (Tris-EDTA) buffer to 50 ng µL−1 final concentration and stored at 4 °C before progressing to downstream PCR applications. Five SSR marker primer pairs for *D. noxia* resistance, which occur on chromosome 7D, viz. *Xgwm44* [60,80], *Xgwm111* [60,80], *Xgwm437* [79], *Xgwm473* [49] and *Xgwm635* [80], were synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Inc. Coralville, Iowa, USA, www.IDTDNA.com) and were provided by Whitehead Scientific PTY (Ltd) Cape Town, South Africa (www.whitesci.co.za). PCR reaction conditions recommended for the KAPA 2X Ready Mix PCR Kit (KAPA Biosystems, Cape Town, South Africa, www.kapabiosystems.com) were applied. Each PCR reaction consisted of 10 µL (1x) KAPATaq 2X Ready Mix, 0.5 µL (10 µM) per SSR primer and the remaining volume (5.0 µL) of DNAse-free water. PCR was performed with a profile comprising initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation involving 95 °C for 30 s, annealing at a specific temperature for individual marker for 30 s, and extension at 72 °C for 30 s. Thereafter, a final extension step of 5 min at 72 °C was performed.

Relevant SSR marker-specific PCR amplicons were separated on 3.0–3.5% (w/v) high resolution agarose gel (Certified Low Range Ultra Agarose, Bio-Rad Laboratories, Inc. Hercules, CA, USA) stained with GelStar™ Nucleic Acid Gel stain (Lonza, Morristown, NJ, USA). Fragment separation was performed in an electrophoresis chamber containing 1x Tris-borate-EDTA (TBE) buffer and run at 100–125 V for 1–4 h. The SSR product sizes were determined according to 100 bp and/or 20 bp DNA ladders (Lonza SimplyLoad R, Lonza, Morristown, NJ, USA). A digital photograph was taken of the gel under UV light exposure with the Bio-Rad Molecular Imager Gel DocTM XR Instrument. Observed SSR marker alleles were sized, recorded and analyzed per cultivar both visually and with Bio-Rad image LabTM gel analysis software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Data for each single example plant (damage rating score and successful marker analysis) are tabulated in Supplementary Table S2. The mean phenotypic damage rating for the five single plants from each accession was used to rank the accessions from most resistant to least resistant, and calculate the standard error of means presented in Table 3.

5. Conclusions

In recent years, the spread of agricultural crop pests has become broader [147,148]. Prediction models [149] estimate that by the middle of this century, many important crop-producing countries will be fully saturated with pests. These authors [149] further state that, in spite of the quarantine and phytosanitary measures that are designed to prevent pest spread, natural dispersal and trade eventually result in invasions of crop pest species into previously pest-free areas. The global redistribution of species is not limited to pests that spread to previously pest-free areas. Virulent biotypes of pests can similarly spread, causing the resurgence of a pest in
an area where it was formerly controlled. Climate change will undoubtedly influence the distribution and pest status of *D. noxia*. A clear understanding of the genetic control of *Dn resistance*, together with robust diagnostic markers, will be important in addressing challenges posed by this aphid in a timely manner.

The landrace origins and proximity of *Dn resistance* gene(s) to the centromere of 7D have been put forward as possible explanations for the difficulties encountered in the search for diagnostic markers for this trait to date. However, following thorough deliberation, it appears that additionally, two inadvertent faults may have blurred the accumulation of a coherent body of information applicable to *Dn resistance*. The “single dominant gene” assessment, initially accepted as the model of genetic control for resistance to this pest, has paradoxically permeated and simplified the underlying assumptions of many studies. This may have hindered critical investigation, despite multiple studies contending that *Dn resistance* is controlled by closely linked genes, multiple alleles at the same locus, or QTL influenced by the genetic background they occur in. Reconsideration of inadvertent assumptions or omissions, with appropriate reflection on the *D. noxia* biotype used to generate the data, may help better understand previous studies and plan future ones. This review calls for a more fastidious approach to the interpretation of results. Should it hold true that the genetic control of *D. noxia* resistance is more complex than originally thought, it could follow that a *D. noxia* biotype-specific R-gene/allele/QTL interaction, or possibly even a *D. noxia* biotype-specific resistance–response pathway interaction, may be at play. This, together with the potential pleiotropic and epistatic effects of genes involved in *Dn resistance*, should be investigated in future studies.

**Supplementary Materials:** Supplementary Materials can be found at http://www.mdpi.com/1422-0067/21/21/8271/s1. Figure S1: Map of South African wheat production regions. Table S1: Genotypic data of individual sample plants including PIC values and orthologs of markers Xgwm44, Xgwm111 and Xgwm437. Table S2: Historic data used to calculate the mean resistance reaction (SEM) to RWASA1 and RWASA2 for the test panel accessions.

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**Abbreviations**

| Abbreviation | Definition |
|--------------|------------|
| ICARDA       | International Center for Agricultural Research in the Dry Areas |
| PIC          | Polymorphism Information Content |
| QTL          | Quantitative Trait Loci |
| RWA1         | Russian wheat aphid, United States of America, original biotype |
| RWASA1       | Russian wheat aphid, South Africa, original biotype |
| RWASA2       | Russian wheat aphid, South Africa, biotype 2 |
| RWASA3       | Russian wheat aphid, South Africa, biotype 3 |
| RWASA4       | Russian wheat aphid, South Africa, biotype 4 |
| RWASA5       | Russian wheat aphid, South Africa, biotype 5 |
| RWASY        | Russian wheat aphid, Syria, original biotype |
| RWATR        | Russian wheat aphid, Turkey, original biotype |
| SSR          | Single Sequence Repeat |
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