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Localisation of Loci Involved in Resistance to *Diaporthe toxica* and *Pleiochaeta setosa* in White Lupin (*Lupinus albus* L.)

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Abstract

L. albus **is an annual grain-legume crop mainly grown for high-protein fodder worldwide but also to produce large seeds for human consumption as a snack-food. In order to make genetic gains in grain yield, assessment of the genetic variation in the germplasm and identification of loci associated with agronomic traits are essential. Phomopsis blight (PB) and Pleiochaeta root rot (PRR), caused by the fungal pathogens** ***Diaporthe toxica*** **and,** ***Pleiochaeta setosa*** **respectively, are two major yield-limiting diseases of the** ***L. albus*** **crop. The extent of genetic diversity in 94 accessions of** ***L. albus*** **comprising: Australian and exotic cultivars, advanced breeding lines, and landraces originating from 26 different countries was determined utilizing PCR-based genic, and microarray-based Diversity Arrays Technology (DArT™), markers. All accessions were evaluated for resistance to PB in two plant tissues (leaves and stems) using either sprayed or injected spore inoculum. A subset of 58 accessions was further evaluated for resistance to PRR by growing seedlings in spore-infested potting mix. The combined data of 724 (50 genic- and 674 DArT) markers were used for cluster analysis. A subset of 324 markers with call rate ≥95% and predicted disease scores of different genotypes were used to identify marker loci accounting for phenotypic variation in PB and PRR resistance using linear regression analysis. Several markers showed significant association with PB or PRR resistance at** $P < 0.05$**. Our results showed that favourable alleles for PB and PRR resistance are present in the diverse accessions investigated and they will provide**

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valuable materials for lupin breeding.

Keywords

***L. albus*, Genetic Diversity, Phomopsis Blight, Pleiochaeta Root Rot, DArT, Linear Regression Analysis, Genome-Wide Association Analysis**

1. Introduction

Sufficient genetic variation in key agronomic traits is very important for the successful genetic improvement of any crop. Several methods based on morphological markers or biochemical attributes (such as isozyme and protein profiles, and molecular markers) have been utilised for the assessment of genetic diversity in a range of agricultural crops including wheat, barley, sorghum, rapeseed, rice, and cotton [1]-[8]. Molecular markers based on chloroplast DNA variation, randomly amplified polymorphic DNA (RAPDs), simple sequence repeat (SSR), inter-simple sequence repeats (SSR), restriction fragment length polymorphism (RFLP), single nucleotide polymorphism (SNPs) and Diversity Arrays Technology (DArT) have been utilised for genetic diversity assessment, molecular mapping, and genome-wide associations in a huge range of plants and animals [9]-[17].

In recent years, several genomic resources have been developed in *Lupinus angustifolius* and other species of the Hologalegin/Dalbergioid and Phaseoloid clades, such as *Medicago truncatula L.*, *Cicer arietinum L.*, *Cajanus cajan* and *Glycine max* [18]-[24]. However, such extensive resources are not yet available for the genetic improvement of *Lupinus albus* L. (2n = 2x = 50, white or broad-leaf lupin) crop.

In *L. albus*, Phan *et al*. [25] developed the first linkage map using 105 intron targeted amplified polymorphisms (ITAPs) and expressed sequence tag simple sequence repeats (EST-SSR), plus 220 AFLP markers in a set of F_8 recombinant inbred lines derived from the cross Kiev Mutant/P27174. Croxford *et al*. [26] also developed 91 sequence-tagged site (STS) markers based on melting curve analysis from EST-derived and genomic libraries of *L. albus*. These marker systems were further utilised for mapping qualitative traits controlled by major genes and quantitative traits controlled by quantitative trait loci (QTL) such as anthracnose resistance, flowering time, alkaloid content and phenology traits [25] [27]. Recently, Raman *et al*. [28] assessed genetic diversity in *L. albus* germplasm accessions using 20 PCR-based markers and 295 DArT markers derived from a microarray chip containing 15,000 clones from the metagenome of 96 diverse accessions of the *Lupinus* crop species: *L. angustifolius*, *L. albus*, *L. luteus L. mutabilis*, *L. cosentinii*, *L. atlanticus*, *L. digitatus*, *L. hispanicus*, *L. princei*, *L. pilosus* and *L. palaestinus*. However, this metagenomic chip had a low representation (7.3%) of *L. albus* accessions (B. Buirchell, pers. comm.). Vipin *et al*. [29] developed a second Diversity Array chip based on 5376 clones of *L. albus*, and 1152 clones each of *L. luteus* and *L. angustifolius* accessions. This enriched array, with 70% (5376/7680) being *L. albus* clones is likely to facilitate a range of molecular analyses required for genetic improvement programs, such as estimation of genetic diversity, and analysis of linkage disequilibrium among *L. albus* populations.

Phomopsis blight (PB) caused by *Diaporthe toxica*, and Pleiochaeta root rot (PRR) disease, caused by the fungus *Pleiochaeta setosa* are major yield limiting diseases of *L. albus* especially in eastern Australia [30]-[32]. Genetic variation for resistance to both fungal pathogens exists within *L. albus* germplasm and has been exploited by lupin breeding programs. The cultivars “Luxor” and “Rosetta” which have improved level of resistance to PRR were developed, and released for commercial cultivation in Australia. Luxor was derived from the cross Lucky-1/Kiev Mutant. PRR-resistance was donated by the original French variety “Lucky”, which is likely to have gained its resistance from Azores landraces. Rosetta originated from the cross P23277/Start, where the female parent is a Ukrainian breeding line (also known as “M-5”) and Start is a Russian variety. Rosetta has lower PRR-resistance than Luxor but greater than the varieties that it superseded (Kiev Mutant and Ultra).

Selective breeding and domestication has exacerbated the genetic bottleneck in several agricultural crops [33]. Therefore, assessment of molecular diversity is of paramount importance in order to broaden the genetic diversity in lupin genetic improvement programs by identifying and subsequently utilising new alleles, including for PB and PRR resistance. In a recent study, Cowley *et al*. [34] identified genomic regions associated with resistance to Phomopsis pod blight using a linkage map of *L. albus* constructed previously [25] [29] from an F_8 re-

combinant inbred line population derived from the cross Kiev Mutant/P27174, where Kiev Mutant was susceptible to PB and P27174 was resistant. Among eight QTL identified, two were consistently detected in two phenotyping environments on LG3 and LG10 and accounted for up to 36.9% of the total phenotypic variance. However, the usefulness of such QTL in the diverse white lupin germplasm is not known yet.

Genome-wide association analysis (also referred as association mapping/Genome-Wide Association Studies) has been utilized to uncover alleles associated with trait(s) of interest in the diverse germplasm based on linkage disequilibrium [35]-[38]. This approach captures global allelic diversity resulted from historical and evolutionary recombination events, whereas linkage mapping exploits allelic diversity present in the parental lines of genetic mapping population. In *L. albus*, Iqbal *et al*. [39] investigated population structure and linkage disequilibrium in 122 accessions obtained from the USDA germplasm collection. Association analysis revealed two markers explaining up to 22.7% of the phenotypic variation for seed weight. In literature, loci associated with both PB (affecting stem and leaf) and PRR have not been reported in the diverse germplasm yet.

The objectives of the present study were to (1) assess the genetic diversity of *L. albus* cultivars, breeding lines and landraces, (2) evaluate phenotypic variation for PB and PRR resistance, and (3) identify loci associated with resistance to *Diaporthe toxica* and *Pleiochaeta setosa* casing PB and PRR, respectively in white lupin. Genetic dissection of loci involved in PB and PRR resistance would enable plant breeders to develop improved selection strategies. Furthermore, knowledge on the extent of genetic diversity is vital to the breeding programs for (1) identifying diverse parental lines that can be utilized for making crosses aimed to exploit heterosis, (2) identifying and introgressing novel alleles to develop superior varieties for target environments, and (3) the curation of new germplasm for future use [8].

2. Materials and Methods

2.1. Plant Material and DNA Extraction

A total of 94 *L. albus* cultivars, advanced breeding lines, and landrace accessions were utilized in this study. A detailed description of these accessions was given in our previous study [28]. Total genomic DNA was isolated as described in [28] and approximately 100 µg/µL was used for DArT genotyping.

2.2. Genotyping and Data Analysis

DArT analysis was conducted at DArT Pty Ltd (Canberra, Australia) using the general procedures described previously [14]. In this study, we utilized the newer, predominantly *L. albus* DArT array. Details of this array design, platform and genotyping, and scoring of dominant DArT markers have been previously described [29]. Binary data derived from the newer *L. albus* array was combined with PCR-based markers and DArT data from the multispecies array generated in our previous study [28]. Monomorphic markers were excluded from subsequent analysis. The samples were grouped into two populations: advanced breeding lines/cultivars (64) and landraces (30). Shannon Information Index (I) and the number of unique alleles were calculated using the GenAIEx program version 6.1 [40]. Binary data matrices were used to calculate allele frequencies and percent polymorphism (PLP), and to estimate gene diversity statistics, including: total gene diversity across the two populations (*Ht*), average gene diversity within populations (*Hs*), and gene diversity between populations (*Dst*). The AFLP-SURV v1.0 program [41] was employed for these calculations. Band richness (*Br*) was calculated using the program AFLPDiv_V1.1 [42].

2.3. Phylogenetic Construction and Principal Component Analysis

Genetic similarity matrices on the basis of Jaccard coefficient [43] were calculated using combined data from genic and DArT markers. Similarity matrices were then used for hierarchical agglomerative cluster analysis to assess relationships among all genotypes. Phenogram was constructed using the weighted pair group method with arithmetic averages (complete linkage method) implemented in the Primer 6 software package [44]. A total of 1000 random permutation tests were performed to obtain statistically significant evidence (at 5% significance level) of genuine clusters in the samples. Ordination of genotypes was conducted for each marker system to visualize the genetic relationships among the accessions by principal component analysis, as implemented in the Primer 6 software package. A 2D plot of the first two principal components (PC1 and PC2) was drawn to visualize the grouping of the genotypes.

2.4. Phenotyping for PB Resistance

The phenotyping of Phomopsis infection in various *L. albus* plant tissues was assessed independently and has been previously described: leaf [45], stem [46] (**Table 1**), and pod [32]. For the data presented here the important points are summarized below. Inoculum was produced using isolate DAR80114 (stored at Living Culture Collection at NSW Department of Primary Industries, Orange, NSW). This isolate was collected from a natural outbreak of Phomopsis blight in 2004 [31]. Spores were washed from pure cultures grown on oatmeal agar and were adjusted to a spores suspension of 5×10^6 spores per mL using a heamocytometer. Spore suspensions were either stored at 4˚C, or were frozen at −18˚C and thawed as required. All inoculum was used on the same day when prepared. Experiments were designed using DiGGer design software [47] and analysed using ASReml-R [48].

Phomopsis stem blight assessed in a glasshouse screening experiment consisted of a split-plot design of three replicates sown in a 20 × 30 pot array. Each replicate (20 × 10) contained two main-blocks of 100 pots (10 × 10 array). Each main-block was split into two sub-blocks (5 × 10 array). Inoculation treatments were assigned to the sub-blocks. There were four sub blocks in each replicate. Two inoculation treatments were used: either a conidial spore suspension of 5×10^6 spores per ml was sprayed to runoff (the plants were enclosed by a large plastic sheet to maintain a dew period for 48 h), or a 0.05 µl aliquot of the prepared spore suspension was injected into each stem between the 2nd and 3rd internodes. Both inoculation treatments were performed on the same day, 40 days after sowing. Lesion length (mm) was measured 28 days after inoculation. Lesion length data was square root transformed before analysis and is hereafter referred to as stemLes. Incidence of pycnidia formation (presence/absence) was visually assessed 98 days after sowing, hereafter referred to as stemPyc.

Phomopsis leaf blight was assessed using detached leaves from glasshouse grown plants. All detached leaf experiments are multiphase-experiments that have separate randomizations and replications for each phase of the experiment, that is, the field component and the laboratory component. Terms for both phases have been fitted in the analyzing model using ASReml-R [48]. The plants were arranged in a randomized complete block in the glasshouse with 3 replicates. Three leaves per plant were removed, placed in sealed bags and transported on ice to the laboratory and inoculated by spraying the spore suspension over the upper surface of the leaves in a laminar flow cabinet. After inoculation, each leaf was then placed on water agar medium (10 g technical agar, Difco) amended with 10 mg kinetin and 0.1 g aueromycin L^{-1} in an individual 9 cm-diameter Petri dish with the lid on but left unsealed to aid scoring. The cut end of the petiole was pressed into the agar medium and the leaflets evenly spread and separated across the dish. The addition of the hormone kinetin helped to delay leaf senescence to avoid confounding natural senescence with symptoms caused by the pathogen. Phomopsis leaf blight was scored 7 days after inoculation using several methods to quantify variation in the disease symptoms:

(a) percentage leaf area affected was assessed by visually determining the amount of necrosis on each leaflet of each leaf. Referred to here as leafPaa;

(b) the number of sporulating pycnidia produced per cm^2 of leaflet surface area was recorded with the aid of a

Table 1. Phenotyping data sets used in this study to detect QTLs via association mapping for resistance/susceptibility to two pathogenic fungi causing disease in *Lupinus albus* (white lupin).

Dataset	Disease	Assay tissue	Spore inoculation method	Genotype number	Disease measure	Label	Reference to screening method or phenotyping results
1	Pleiochaeta Root Rot	Seedling root	Potting mix	58	Lesion severity[$]	N/A	30
2	Phomopsis	Stem	Spray	94	Lesion length[*]	stemLes (spray)	31
3	Phomopsis	Stem	Spray	94	Pycnidia incidence	stemPyc (spray)	31
4	Phomopsis	Stem	Inject	94	Lesion length[*]	stemLes (inject)	31
5	Phomopsis	Stem	Inject	94	Pycnidia incidence	stemPyc (inject)	31
6	Phomopsis	Detached leaf	Spray	94	Lesion severity	leafLes	32, 46
7	Phomopsis	Detached leaf	Spray	94	Pycnidia incidence[$]	leafPyc	32, 46
8	Phomopsis	Detached leaf	Spray	94	Percent leaf area affected	leafPaa	32, 46

[*]lesion length (mm) was square-root transformed prior to analysis. [$]Previously unpublished phenotyping data. N/A: Not Applicable.

stereomicroscope. Referred to here as leafLes; and,

(c) pycnidia formation on leaflets scored as incidence (*i.e.*, present or absent), hereafter referred to as leafPyc. The phenotyping datasets are listed in **Table 1**.

2.5. Phenotyping for PRR Resistance

The phenotyping methods for PRR in *L. albus* have been previously described [30]. The important features are summarised here. A single-spore isolate of the disease-causing fungus *Pleiochaeta setosa* (PS6) was grown in sealed Petri dishes containing 20% V8 media plus 5.4 g of $CaCO_3$ and 20 g of agar L^{-1}. The plates were maintained with a 12-hr photoperiod at 19˚C. After 21 days the fungus was sub-cultured onto V8 media to initiate conidiospore production. After another 21 days, the spores were mechanically washed from the dishes with sterile water (including 0.1% Wettasoil®). The spore suspension was adjusted so that when mixed with a known volume of potting mix (see below) the final concentration was 1000 spores g^{-1}. In all screening experiments two standard 'control' genotypes were included: P25758 (a very-resistant landrace), and Kiev Mutant (a highly susceptible cultivar from Ukraine) [30] [49] [50].

The PRR scoring system was based on lesion severity on the main root of seedlings that had germinated and grown in the inoculated potting mix for 21 days. The scoring scale was either 0 - 9 (where 0 = no lesion, and 9 = a lesion that has completely severed the root) [30], or 0 - 5 (where 0 = no lesion, and 5 = root severance). Small lateral roots were ignored. In some experiments, each seedling was assessed for lesion severity on the 0 - 9 or 0 - 5 scale within each centimeter from the soil surface for the first 6 cm—resulting in six scores per seedling. Very susceptible genotypes often had the seedling root severed within the first 1 or 2 cm. For such seedlings, all root position scores below the point of severance were recorded as "9" or "5" (depending on which scale was being used). In some other experiments, each seedling root was scored only for the single most severe lesion present in the whole of the top 6 cm of the root.

Experiments consisted of either four replicate pots of each genotype; or two replicate pots. In both cases, 16 seedlings were grown per pot (175 mm diameter, 3.8 L volume) in a regular grid. Experimental designs were generated using DiGGer software [47] [51] in R [52] which allowed the pots to be spatially arranged in replicates, rows and columns in an optimal fashion.

A spore concentration of 1000 g^{-1} of soil was achieved by adding the calculated spore number to a measured mix of moist 80% double-washed river sand and 20% brick sand in a concrete mixer for 90 sec. All handling of pots and potting mix was done under cool, shaded conditions to maximize spore survival. Pots were not given any fertiliser or Rhizobia because seedlings were only grown for 21 days and seed reserves, combined with base-level potting mix fertility, was sufficient for good, rapid growth. All experiments were conducted in a controlled-environment growth room (16-hr photoperiod, 15˚C day, 10˚C night). Pots were arranged in large stainless-steel tubs which enabled the pots to be given extended bottom watering after sowing to ensure uniformity of germination and growth, and to encourage good infection. Tubs were drained of water three days after sowing. Pots were then hand watered from above as required. After 21 days seedlings were carefully removed from soil, rinsed, and scored.

Data from seven phenotyping experiments were combined; they included 58 of the 94 genotypes in the albus diversity set. The phenotype of the remaining 36 genotypes is unknown (although experience tells us that the majority will be susceptible). All data analysis was conducted using R software [52] and the ASREML package [48]. The packages lattice [53], plyr [54], and reshape2 [55] were also used to manipulate and plot the data in R. Data on the 0 - 5 scale were rescaled to 0 - 9 by multiplying by 9/5. Data with six scores per root was collapsed by choosing the highest single lesion score for each seedling. The resulting data was analysed by mixed modelling approach to derive estimated genotype means for lesion score.

2.6. Genotype Association Analysis

The combined dataset of marker scores: PCR-based, meta-genomic *Lupinus* array [28], and the newer tri-genomic *L. albus* DArT array, were imported into the SVS suite version 7 software as per the manufacturer's instructions (Golden Helix). Genotype predicted means of PB and PRR resistance scores (phenotypes) were also imported and used in association mapping analysis. A subset of 324 marker alleles with a call rate ≥95% was selected for trait association between markers and phenotypes. Genome-wide association (GWA) was tested using the linear regression analysis implemented in the SVS suite. The kinship within tested genotypes was analysed

using the "Identity by Descent Algorithm". In order to reduce spurious association between the trait and marker, correction for stratification was made using principal components (PC1 to PC5), which account for population structure. Linear regression analysis was performed using additive model in order to determine marker loci accounting for phenotypic variation for resistance to PB and PRR. Data for each marker were normalized by its theoretical standard deviation under Hardy-Weinberg's equilibrium. The map locations of molecular markers that exhibited significant associations with PB and PRR resistance were identified in the existing linkage map of *L. albus* [25] [29] [34].

3. Results

3.1. Allelic Variation among Genotypes

Genetic diversity indices for both PCR-based markers and DArT markers (combined from both meta-genomic and tri-genomic *Lupinus* microarrays) were compared. Genic markers had a high Shannon's Information Index and percent polymorphic loci compared to the DArT markers (**Table 2**). Landraces exhibited higher polymorphism and Shannon's Information Index (*PLP* = 84.3%; *I* = 0.45) and gene diversity (*Hs* = 0.31) than cultivars/advanced breeding lines (*PLP* = 75.3%; *I* = 0.41; *Hs* = 0.27). The cultivars/advanced breeding lines contained six unique alleles and the landrace population possesses five unique alleles. Genic markers also revealed higher overall genetic diversity indices: total gene diversity (*Ht* = 0.34); mean gene diversity within population (*Hs* = 0.31) compared with DArT markers (*Ht* = 0.3 and *Hs* = 0.27). Nine (*Gst* = 0.09) and eleven percent (*Gst* = 0.11) differentiation in the population was identified with DArT and genic markers, respectively (**Table 3**).

3.2. Genetic Similarity and Phylogenetic Relationships among Genotypes

The genetic similarities (GS) values for all markers ranged from 20.7% to 77%. The highest GS was observed between the accessions ESTA1 and FP21, and the lowest was between P28991 and Kiev Mutant. A phenogram based upon the 724 DArT and PCR-based markers showed a higher level of genetic diversity in the germplasm collection (**Figure 1**). At the 20% of GS, none of the accessions could be differentiated from each other. However, at 40% of GS, accessions could be grouped into six well-differentiated clusters (**Figure 1**). Not all accessions grouped according to their country of origin.

Two accessions P27662 and P28233 originated from Turkey and Ethiopia, respectively did not show any grouping with other accessions. In cluster I, four accessions P28991, WK320, P27441, and WK302 were grouped

Table 2. Genetic diversity of breeding lines (BL), cultivars (CV) and landraces of *L. albus*. *n* = population size; *I* = Shannon's Information Index; *PLP* = % of polymorphic loci at 5% level; *Br* = band richness adjusted to the smallest group size; Unique alleles appear in only single population; and Hs = Nei's gene diversity index. Values in parentheses are the standard errors of the individual estimates.

Population	Diagnostic Statistic (for each marker class)									
	n	*I*		*PLP*		*Br*		Unique allele number		*Hs*	
		Genic	DArT	Genic	DArT	Genic	DArT	Genic	DArT	Genic	DArT
BL/CV	64	0.45 (0.03)	0.37 (0.01)	81.6	69.0	1.9	1.9	4	2	0.30 (0.02)	0.23 (0.01)
Landraces	30	0.45 (0.03)	0.45 (0.01)	83.7	84.9	1.8	1.97	1	4	0.31 (0.02)	0.3 (0.01)

Table 3. Summary statistics of gene diversity of *L. albus* germplasm. *Ht* = Total gene diversity; *Hs* = mean gene diversity within populations; *Dst* = average gene diversity between population groups; and *Gst*, =relative differentiation of populations (standard error given in parentheses).

Marker system	*Ht*	*Hs*	*Dst*	*Gst*
Genic	0.34	0.31 (0.00)	0.03 (0.00)	0.11 (0.01)
DArT	0.3	0.27 (0.03)	0.03 (0.00)	0.09 (0.11)

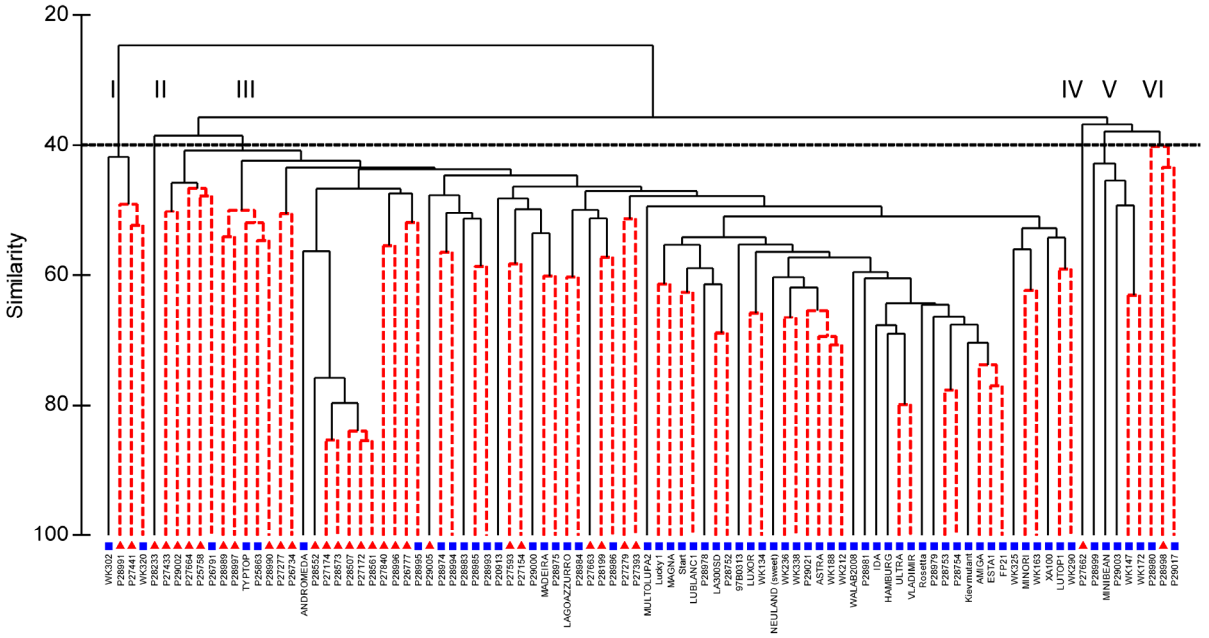

Figure 1. Phenogram from WPGMA (weighted pair group method, arithmetic mean) based on 724 PCR-based and DArT markers, using Jaccard's coefficient, showing similarities among 94 *L. albus* accessions representing different lineages. Dotted lines indicate the genotypes that do not differ statistically at $P = 5\%$ level of significance, following 1000 permutation tests implemented in Primer 6. The x-axis represents the full set of genotypes, and the y-axis defines a similarity level (0 - 100) at which samples are considered to be grouped. The groups (in roman letters I to VI) separated at a 40% similarity threshold (dotted line) are indicated. ▲ (triangles) and ■ (squares) represent landrace accessions, and cultivars/breeding lines respectively.

together. Cluster III represented a mix of 80 accessions representing landraces, advanced breeding lines, and cultivars. In this cluster, subpopulation of landrace accessions P28561, P27174, P28573, P28552, P27172, and P28507 (all originating from Ethiopia) were present. As reported previously in Raman *et al*. [28], both Western Australian-bred genotypes, cv. Andromeda and breeding line WALAB2008, which are derived from Ukrainian parent (cv. Kiev Mutant) and anthracnose-resistant Ethiopian parent (P27174) were not tightly clustered together; however, both, along with Kiev Mutant, were grouped in the cluster III. Cluster V had exclusively advanced breeding lines and cultivars. For example, the Australian lupin varieties and breeding lines are clustered in the same group (97B0313, Luxor and WK134). Cluster V1 contained three accessions P28980, P28998 and P29017 originated from Russia, Spain, and Poland, respectively.

The PCO analysis was used to determine overall genetic diversity and population structure within 94 accessions used in this study. The first four principal components explained approximately 28.8% of the observed genetic variation and revealed weak grouping (structure). Accessions could be grouped into four (I - IV) subpopulations. The first subpopulation consisted a majority of advanced breeding lines and cultivars which represented accessions grouped in Cluster 1 (**Figure 2**). Subpopulation II consisted of a mixture of advanced breeding lines/cultivars, and landraces that showed grouping in cluster II, IV, and VI. Subpopulations III consisted a majority of landrace accessions. However, there was some representation of advanced breeding lines such as P26971 which showed grouping with P27433 landrace accession from Syria. In cluster IV, accessions were predominantly from landraces such as P28507, P28573, P2856, P27172, and P27174. All these accessions were originated from Ethiopia. PCO analysis based on DArT markers showed very strong clustering of four genotypes located some distance from the rest (see also **Figure 1**, clade I). The extreme group of four seems surprising based on our knowledge of their origins: P27441 = a Syrian landrace; P28991 = a Polish landrace; WK302 = an Australian breeding line from a complex pedigree involving German and French cultivars with Russian and Ukrainian landraces; and WK320 = an Australian breeding line from another complex pedigree involving a Greek landrace and UK breeding lines. These comments illustrate the broad range of genetic material already being exploited in *L. albus* breeding programs.

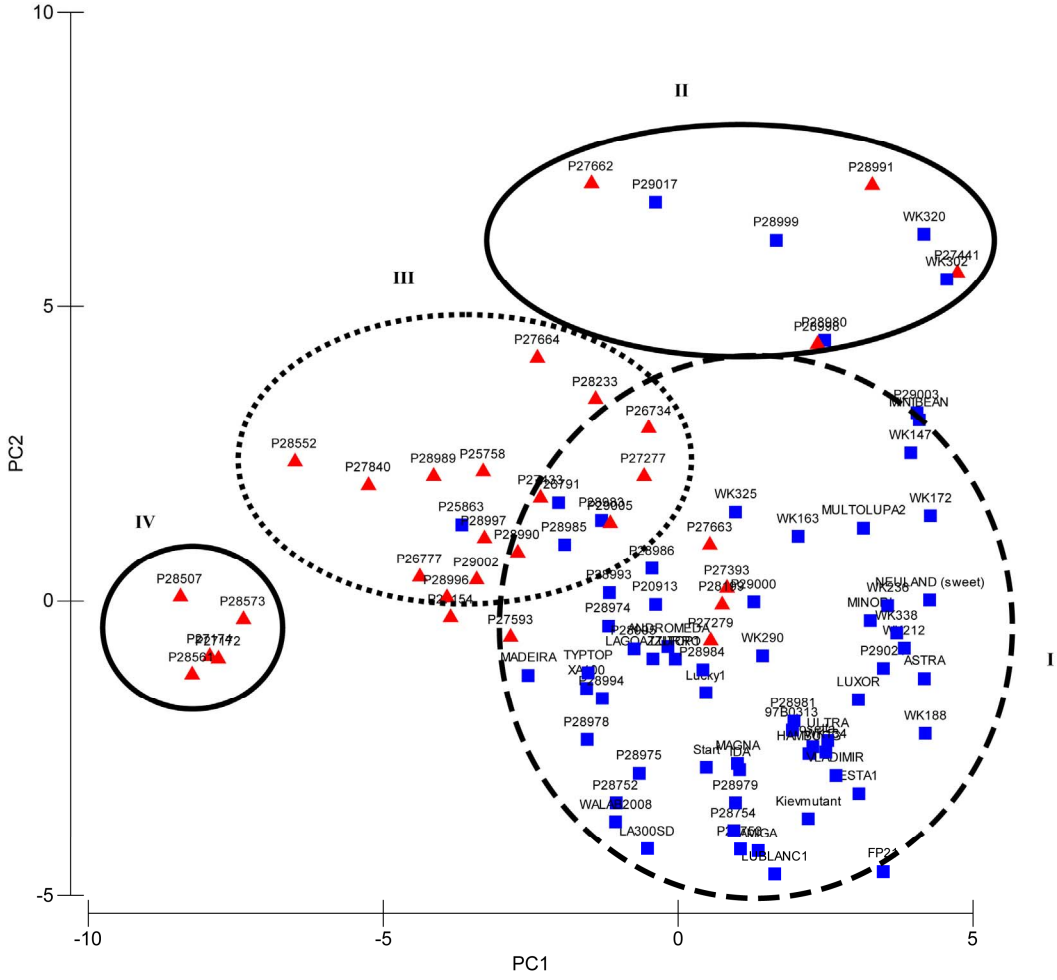

Figure 2. Principal component analysis of 94 accessions of *L. albus* (see **Table 1**, in Raman *et al*. [28] based upon 724 markers. Subpopulations were labeled as I to IV based upon their grouping patterns. Axis (dimension)-1 and axis-2 explained 11.7% and 7.6% of the genetic variation, respectively. ▲ (triangles) and ■ (squares) represent cultivars/breeding lines, and landrace accessions, respectively.

3.3. Phenotypic Variation for PB Resistance

Genetic variation exists for resistance to Phomopsis stem blight with significant ($P < 0.001$) effects of genotype, inoculation and their interaction on lesion length score and pcynidia formation ($P < 0.05$). There was a weak but significant correlation between the two scores (**Figure 3**). For stemLes (spray) the most resistant genotypes were breeding lines P28979, P28978, and P28975 from the Ukraine. P28096 (landrace from Syria) and P27664 (landrace from Turkey) were also resistant. The predicted mean of Kiev Mutant and Rosetta were 2.68 and 6.35, respectively. All of the *L. albus* cultivars grown commercially in Australia had similar incidences of pycnidia formation in the spray treatment. Genetic variation also exists for Phomopsis leaf blight using each of the methods to measure the disease severity [32] [45]. The range for leafPaa was 7.1 to 65.8, although the average LSD (5%) between genotype means was large (28.8) [45].

3.3. Phenotypic Variation for PRR Resistance

Dot plot of the predicted means for lesion scores clearly showed genetic variation for resistance to PRR in different accessions of *L. albus* (**Figure 4**). Genotype fixed effects for PRR lesion score were highly significant ($P < 0.001$). Random effects of pot, range, and row were accounted for within each experiment. The average LSD (5%) between genotype means was 1.489 (on the 0 - 9 scale).

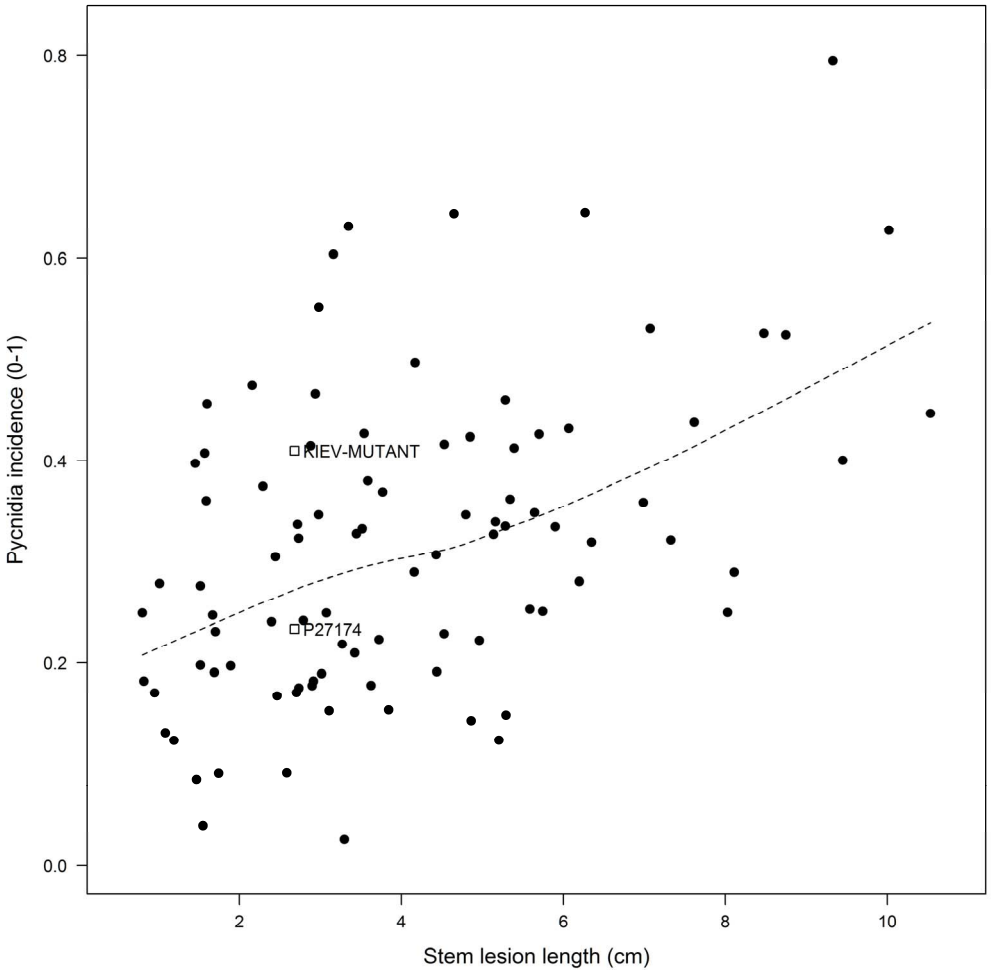

Figure 3. Predicted genotype means for Phomopsis stem blight phenotyping using two scoring systems on 94 lupin genotypes. These data are previously unpublished. The two labeled genotypes were extremes when tested for pod Phomopsis blight on the same genotypes (as described previously [31]). The loess line of best fit shows the weak but significant correlation (r = 0.47) between these two variables.

3.4. Genome-Wide Association Analysis for PB and PRR Resistance

A total of 324 marker loci with a call rate ≥95% were chosen for the detection of genome-wide association between markers and phenotypes (PB or PRR resistance). Ten principal components which explained 46.3% variation were used to correct stratification in the population structure. Linear regression analysis showed that 65 markers showed association with PB resistance (**Table 4**). DArT marker lPb-515985 (derived from the meta-genomic array) showed a highly significant association ($P < 0.0001$) with resistance to PB when measured with the PB stem lesion injected method. The same marker also showed significant association with resistance to PB when disease lesions were as scored as stemPyc. Of the 65 markers, 15 showed association with more than one measure of PB resistance. For example, PCR-based genic marker Lup272a and DArT marker lPms-527315 showed significant association with PB resistance evaluated on the basis of leafPaa, and leafLes (spray) (**Table 4**). DArT marker, lPms-524453 showed a significant association ($P = 0.01$) with stemPcy (spray).

In the subset of 58 accessions, DArT markers lPb-462333, lPms-749715, lPb-449899, Ipms-527182, Ipb-460448 and Ipb-449180 were significantly ($P < 0.005$) associated with resistance to PRR (**Table 5**). DArT markers lPb-462333 and lPb-449899 were derived from meta-genomic microarray, whereas DArT marker lPms-749715 was derived from the tri-genomic microarray. None of these markers were mapped on the genetic linkage map of *L. albus* generated in the previous studies [25] [29]. In addition, 29 markers were suggestively significant ($P < 0.05$) with PRR resistance (**Table 5**); one ITAP marker LUP273 was mapped on the linkage group

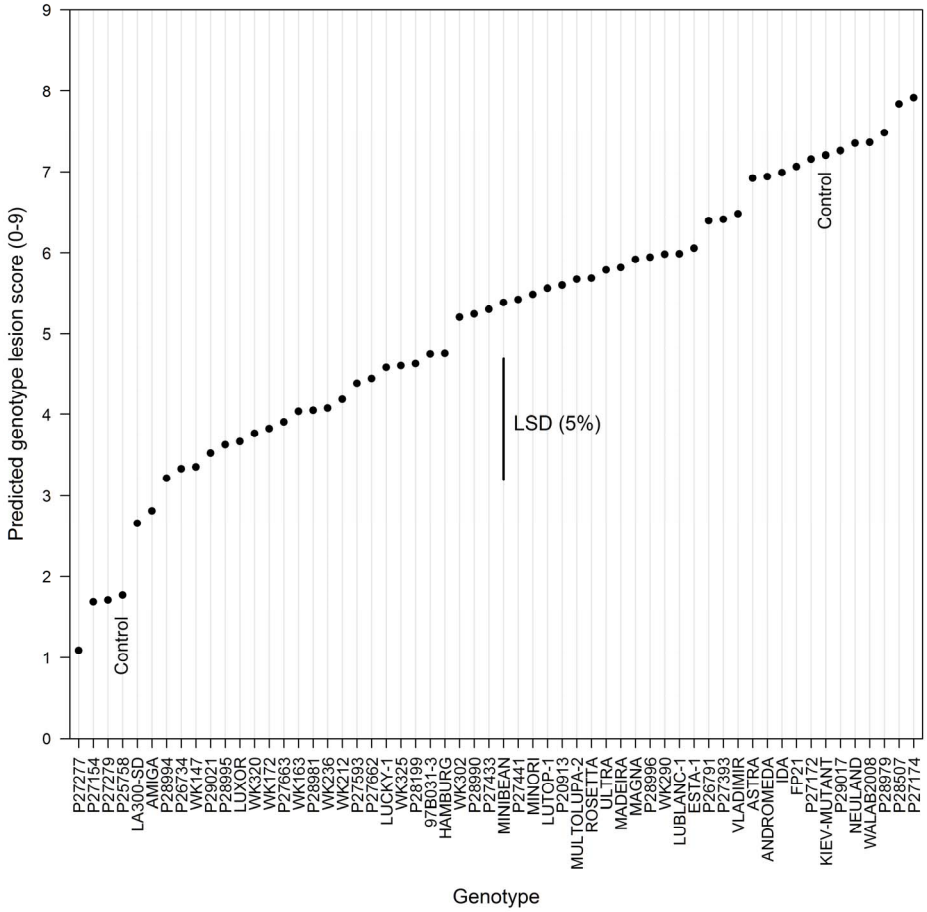

Figure 4. Dot plot of the predicted genotype means for Pleiochaeta Root Rot lesion score on *L. albus* seedlings grown in controlled-environment experiments (dataset number 1, **Table 1**). The mean LSD (at $P = 0.05$) between genotype means is shown as a vertical bar. The standard "control" genotypes used in each experiment are labeled (P25758 = resistant, Kiev Mutant = susceptible).

16 [25] [29].

4. Discussion

L. albus is a diploid, self-pollinated, grain legume, crop species which is well-adapted to a range of environments world-wide. Disease resistance to *P. setosa* and *D. toxica* is of paramount importance for the *L. albus* breeding programs which are developing high-yielding, high quality cultivars for stock-feed and human consumption. The other very important disease resistance needed in most *L. albus* growing regions is anthracnose resistance (caused by the fungus *Colletotrichum gloeosporioides*) [56]. Fortunately, resistance to anthracnose has been identified in Ethiopian landraces, and successfully introgressed into adapted backgrounds in Australia.

Our results have clearly shown that substantial genetic diversity, including for PB and PRR resistance, is present in the 94 lupin accessions investigated. Cluster and PCO analyses showed that there was a weak association between grouping (structure) of genotypes and their country of origin (**Table 1**, (28), **Figure 1** & **Figure 2**) Phenograms based on PCR-based genic and DArT markers showed some inconsistent relationships (original data not shown), as they relied on different number of marker alleles with variable genomic coverage. Furthermore, different marker systems (genic and DArT) are known to provide different estimates of genetic diversity, as marker analysis is dependent upon the evolutionary parameters of the underlying DNA sequence variation [57]. Recently, Vipin *et al.* [29] reported that both genic and DArT markers map on different genomic regions of the lupin genome (linkage groups constructed in an F_8 recombinant inbred population derived from Kiev Mutant/P27174). This suggests that different markers may capture variable level of genetic variation, particularly

Table 4. Genome-wide association analysis showing marker loci explaining significant ($P < 0.05$) linkage with PB resistance in *L. albus* accessions. The rows in bold are markers that showed consistent association with more than one measure of PB resistance. Chromosomal location of markers is based on the map position onto the linkage map constructed previously [29].

Inoculated tissue	Phenotyping measure	Inoculation method	Marker locus	#Regression −log10 P	Chromosomal Location
Stem	stemLes	Spray	CHS9a	1.928	Unknown
Stem	stemLes	Injection	lPb-199523	2.913#	Unknown
Stem	stemPyc	Spray	lPb-329051	2.713#	Unknown
Leaf	**leafLes**	**Spray**	**lPb-329590**	**1.725**	**Unknown**
Stem	**stemPyc**	**Injection**	**lPb-329590**	**1.754**	**Unknown**
Leaf	leafPaa	Spray	lPb-331912	1.875	Unknown
Stem	stemLes	Spray	lPb-332222	3.168#	Unknown
Stem	stemLes	Injection	lPb-332288	2.998#	Unknown
Leaf	leafPyc	Spray	**lPb-333243**	**1.579**	**Unknown**
Leaf	leafLes	Spray	**lPb-333243**	**2.336**	**Unknown**
Leaf	**leafPaa**	**Spray**	**lPb-333243**	**1.797**	**Unknown**
Stem	stemLes	Spray	lPb-333295	1.582	Unknown
Stem	stemLes	Injection	lPb-449022	2.021	Unknown
Leaf	leafLes	Spray	**lPb-449248**	**1.570**	**Unknown**
Leaf	**leafPaa**	**Spray**	**lPb-449248**	**1.767**	**Unknown**
Stem	stemLes	Injection	lPb-449359	2.918#	Unknown
Stem	stemLes	Injection	lPb-449397	1.970	Unknown
Stem	stemPyc	Injection	lPb-449737	2.442	Unknown
Stem	**stemLes**	**Injection**	**lPb-449904**	**1.999**	**Unknown**
Stem	**stemPyc**	**Spray**	**lPb-449904**	**2.548#**	**Unknown**
Leaf	leafPyc	Spray	lPb-460200	2.008	Unknown
Stem	stemPyc	Spray	lPb-460248	1.560	Unknown
Stem	stemPyc	Spray	lPb-462552	1.902	Unknown
Stem	**stemLes**	**Spray**	**lPb-462744**	**2.045**	**Unknown**
Stem	**stemPyc**	**Spray**	**lPb-462744**	**2.128**	**Unknown**
Stem	stemPyc	Injection	lPb-515431	3.095#	Unknown
Leaf	**leafLes**	**Spray**	**lPb-515485**	**1.823**	**LG10**
Leaf	**leafPaa**	**Spray**	**lPb-515485**	**1.962**	**LG10**
Stem	stemLes	Injection	lPb-515644	1.704	Unknown
Stem	**stemLes**	**Injection**	**lPb-515985**	**6.377#**	**Unknown**
Stem	**stemPyc**	**Spray**	**lPb-515985**	**2.068**	**Unknown**
Leaf	leafLes	Spray	lPb-516988	1.763	Unknown
Stem	stemLes	Injection	lPb-517108	1.573	Unknown
Stem	stemLes	Spray	lPb-524639	1.713	Unknown
Stem	stemPyc	Injection	lPb-524815	1.623	Unknown
Stem	stemLes	Spray	lPms-515231	1.953	LG13-I
Leaf	leafPyc	Spray	lPms-515287	1.631	Unknown
Stem	stemPyc	Spray	lPms-515316	2.057	Unknown
Stem	stemPyc	Injection	lPms-515431	2.917#	Unknown
Stem	stemPyc	Injection	lPms-515465	2.512#	Unknown

Continued

Leaf	**leafLes**	**Spray**	**lPms-515485**	**1.962**	**LG10**
Leaf	**leafPaa**	**Spray**	**lPms-515485**	**2.060**	**LG10**
Stem	stemLes	Injection	lPms-515786	1.599	Unknown
Stem	stemPyc	Injection	lPms-518014	1.647	Unknown
Leaf	leafPyc	Spray	lPms-522563	2.353	Unknown
Leaf	**leafLes**	**Spray**	**lPms-522563**	**1.906**	**Unknown**
Leaf	**leafPaa**	**Spray**	**lPms-522563**	**2.263**	**Unknown**
Stem	stemPyc	Spray	lPms-524453	1.967	LG12
Stem	stemLes	Spray	lPms-524639	1.713	Unknown
Leaf	**leafLes**	Spray	**lPms-524687**	**1.696**	**LG2-II**
Leaf	**leafPaa**	**Spray**	**lPms-524687**	**1.823**	**LG2-II**
Stem	**stemLes**	**Spray**	**lPms-524687**	**1.580**	**LG2-II**
Stem	**stemPyc**	**Spray**	**lPms-524687**	**3.472#**	**LG2-II**
Leaf	**leafLes**	**Spray**	**lPms-527315**	**1.606**	**Unknown**
Leaf	**leafPaa**	**Spray**	**lPms-527315**	**1.785**	**Unknown**
Stem	**stemLes**	**Injection**	**lPms-527365**	**1.942**	**Unknown**
Stem	**stemPyc**	**Spray**	**lPms-527365**	**1.951**	**Unknown**
Stem	stemLes	Spray	lPms-532960	1.856	Unknown
Stem	stemPyc	Spray	lPms-749081	1.583	Unknown
Stem	stemLes	Injection	lPms-749267	2.245	Unknown
Leaf	**leafPyc**	**Spray**	**lPms-749738**	**1.583**	**Unknown**
Stem	**stemPyc**	**Spray**	**lPms-749838**	**1.724**	**Unknown**
Stem	stemLes	Injection	lPms-750115	1.703	Unknown
Leaf	leafLes	Spray	lPms-750356	1.561	Unknown
Stem	stemPyc	Injection	lPms-750752	2.825#	Unknown
Stem	stemLes	Spray	lPms-750903	1.593	Unknown
Stem	**stemLes**	**Spray**	**lPms-750906**	**2.045**	**Unknown**
Stem	**stemPyc**	**Spray**	**lPms-750906**	**2.128**	**Unknown**
Stem	stemLes	Spray	lPms-751073	2.034	Unknown
Leaf	leafPaa	Spray	lPms-751227	1.580	Unknown
Leaf	leafLes	Spray	lPms-751465	1.624	Unknown
Stem	stemLes	Injection	lPms-751519	2.223	Unknown
Stem	stemLes	Spray	lPms-751637	1.638	LG29
Stem	stemPyc	Injection	lPms-751770	2.442	LG13-I
Stem	stemPyc	Injection	LSSR14a	1.553	Unknown
Leaf	leafPyc	Spray	Lup104b	2.312	Unknown
Stem	**stemLes**	**Spray**	**Lup109f**	**2.047**	**Unknown**
Stem	**stemPyc**	**Spray**	**Lup109f**	**1.756**	**Unknown**
Stem	stemLes	Injection	Lup264a	2.081	Unknown
Leaf	leafPaa	Spray	Lup269a	1.705	Unknown
Leaf	**leafPyc**	**Spray**	**Lup272a**	**2.188**	**Unknown**
Leaf	**leafLes**	**Spray**	**Lup272a**	**2.647#**	**Unknown**
Leaf	**leafPaa**	**Spray**	**Lup272a**	**2.762#**	**Unknown**
Stem	**stemPyc**	**Spray**	**Lup272a**	**1.565**	**Unknown**
Stem	stemPyc	Spray	Lup272b	2.215	Unknown
Stem	stemLes	Spray	Lup273	1.924	LG16

[#]Significant association at *P* values < 0.005.

Table 5. Genome-wide association analysis showing marker alleles explaining significant linkage with PRR resistance in *L. albus* accessions. The rows in bold are markers which showed highly significant associations at $P < 0.05$. Chromosomal location of markers is based on the map position onto the linkage map constructed previously [25] [29].

Marker	Regression $-log10\ P$	#Chromosomal Location
lPb-462333	**3.807**	**Unknown#**
lPms-749715	**3.526**	**Unknown#**
lPb-449899	**3.022**	**Unknown#**
lPms-527182	**2.633**	**Unknown#**
lPb-460448	**2.589**	**Unknown#**
lPb-449180	**2.527**	**Unknown#**
lPms-751785	2.290	Unknown
lPb-449532	2.270	Unknown
lPb-515569	2.119	Unknown
Lup273	2.117	LG16
lPb-460089	2.099	Unknown
lPb-462552	2.081	Unknown
lPms-515485	2.043	LG10
lPb-518139	2.021	Unknown
lPms-751131	1.934	LG3
lPms-750988	1.882	Unknown
lPms-527083	1.863	LG3
lPms-748977	1.801	Unknown
Ant2	1.773	Unknown
lPms-518014	1.755	Unknown
lPms-515569	1.741	Unknown
PT1c	1.721	LG21
Lup125a	1.711	LG27
lPb-515485	1.653	Unknown
Lup229TasIa	1.611	LG5
lPms-525217	1.597	Unknown
lPb-333243	1.569	Unknown
lPb-460200	1.559	Unknown
lPms-515166	1.523	Unknown
lPb-448847	1.519	Unknown
lPb-524719	1.508	Unknown
PT1b	1.478	LG21
lPb-517242	1.474	Unknown
CHS9b	1.469	Unknown

#Significant association at P values < 0.005.

when marker coverage along the genome is not large enough. The 724 markers analysed here are expected to provide much better genome coverage and hence resolution of genetic diversity. Newer technologies based on genotyping-by-sequencing are becoming available in several plant species and are expected to complement or even replace the traditional PCR- and DNA hybridization based assay in the near future.

In this study, we have identified genetic diversity among the landraces, cultivars and breeding lines that show a range of phenotypic variation for a range of agronomically desirable attributes such as alkaloid content, and resistance to PRR, anthracnose, and Phomopsis [32] [34] [45] [58] [59]. Genotypic marker data was further employed to develop trait-marker association using the genome-wide association approach [60]. This analysis is known to be influenced by a number of factors, such as population size, mode of reproduction, selection history, genetic-related- ness, and population stratification. In this study, we used small number of accessions (58 to 94) and small genotypic dataset for testing association between markers and resistance to PB and PRR, which will have a limited power of QTL detection. Nevertheless, we have identified several genomic regions associated with resistance to PB which was evaluated on the basis of seven different phenotypic measures. Some of these measures have been used previously in order to identify resistant sources in *L. albus* germplasm. However, the genetics underlying these measures of resistance is not fully understood. Cowley *et al*. [34] have reported that genetic inheritance underlying PB resistance is complex and at least eight genomic regions were associated with PB resistance evaluated on detached pods. In this study, we have identified at least one genomic region delimited with marker lPms-524453 which also showed association with resistance to pod PB on linkage group 12 [34]. This finding suggested that at least some common genomic region control resistance at the stem, and pod stages. Previously, no correlation between the pod and the stem scores has been reported [32] [45].

A smaller training population comprising 58 of the 94 diverse accessions was also used to identify at least six markers significantly associated with PRR resistance (**Table 5**). We were unable to validate these results as loci associated with PRR resistance have not been tagged using molecular markers in *L. albus*. Molecular marker data can be used to estimate the genomic breeding values of additional germplasm (including breeding lines) without the need for extensive phenotyping. Our preliminary association results needs to be validated in the bi-parental population(s). In this study, we have not tested different regression models for trait-marker association due to the small size of population and limited coverage of genome-wide markers. The robustness of trait-marker associations may be tested using different models accounting both population structure and coefficient of relatedness as proposed in previous studies [39] [60]. Further research is required to convert DArT markers into cost-effective and simple PCR-based markers for marker-assisted selection, and then to test their linkage in a range of breeding germplasm.

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