Construction of a high density linkage map and genome dissection of bruchid resistance in zombi pea (\textit{Vigna vexillata} (L.) A. Rich)

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Zombi pea (\textit{Vigna vexillata}) is a legume crop that is resistant to several biotic and abiotic stresses. \textit{Callosobruchus maculatus} and \textit{Callosobruchus chinensis} are serious stored-insect pests of legume crops. We constructed a high-density linkage map and performed quantitative trait loci (QTLs) mapping for resistance to these insect species in zombi pea. An F\textsubscript{2} population of 198 individuals from a cross between ‘TVNu 240’ (resistant) and ‘TVNu 1623’ (susceptible) varieties was used to construct a linkage map of 6,529 single nucleotide polymorphism markers generated from sequencing amplified fragments of specific loci. The map comprised 11 linkage groups, spanning 1,740.9 cM, with an average of 593.5 markers per linkage group and an average distance of 0.27 cM between markers. High levels of micro-synteny between \textit{V. vexillata} and cowpea (\textit{Vigna unguiculata}), mungbean (\textit{Vigna radiata}), azuki bean (\textit{Vigna angularis}) and common bean (\textit{Phaseolus vulgaris}) were found. One major and three minor QTLs for \textit{C. chinensis} resistance and one major and one minor QTLs for \textit{C. maculatus} resistance were identified. The major QTLs for resistance to \textit{C. chinensis} and \textit{C. maculatus} appeared to be the same locus. The linkage map developed in this study will facilitate the identification of useful genes/QTLs in zombi pea.

The genus \textit{Vigna} is an important plant taxon containing nine cultivated legume crops, including mungbean [\textit{Vigna radiata} (L.) Wilczek], blackgram [\textit{Vigna mungo} (L.) Hepper], cowpea [\textit{Vigna unguiculata} (L.) Walp.], rice bean [\textit{Vigna umbellata} (Thunb.) Ohwi and Ohashi], Bambara groundnut [\textit{Vigna subterranea} (L.) Verdc.], azuki bean [\textit{Vigna angularis} (Ohwi) Ohwi and Ohashi], moth bean [\textit{Vigna aconitifolia} (Jacq.) Marechal], zombi pea [\textit{Vigna vexillata} (L.) A. Rich] and creole bean (\textit{Vigna reflexo-pilosa} Hayata) which are grown in various cropping systems on over 25 million hectares in Asia, Africa, Australia and America\textsuperscript{1,2}. These \textit{Vigna} crops are generally susceptible to biotic and abiotic stresses\textsuperscript{1}. Among the \textit{Vigna} species, zombi pea (\textit{Vigna vexillata}) is of particular interest. It is a pan-tropical herbaceous legume plant. Wild zombi pea is distributed widely throughout tropical and sub-tropical regions of Africa, Asia, Australia and America\textsuperscript{3}. The species often develops storage roots that are edible and consumed by people in Africa, India, Australia and Southeast Asia\textsuperscript{4-6}. Edible tubers of \textit{V. vexillata} have a ~15% protein content, which is much greater than the contents of potato, yam and cassava\textsuperscript{7}. Moreover, the yield of \textit{V. vexillata} tuberous roots can be as high as 7 tons per hectare\textsuperscript{8}. The cultivated form of \textit{V. vexillata} is found in a limited area of Africa and Asia (Bali, Indonesia) in which both seeds and tuberous roots are consumed\textsuperscript{9}.

Owing to its wide distribution, \textit{V. vexillata} has adapted to several climatic and environmental conditions, such as infertile\textsuperscript{9}, alkaline\textsuperscript{9}, acidic\textsuperscript{10}, and saline soil\textsuperscript{11}, as well as drought\textsuperscript{11} and waterlogging\textsuperscript{12}. In addition, it is resistant or tolerant to several insect pests and their related diseases, such as \textit{Callosobruchus maculatus}\textsuperscript{13}, \textit{Zabrotes subfasciatus}\textsuperscript{13}, \textit{Maruca testulalis}\textsuperscript{14}, \textit{Maruca vitrata}\textsuperscript{15}, \textit{Clavigralla tomentosicollis}\textsuperscript{15}, and cowpea mottle carmovirus\textsuperscript{16}. The successful hybridization between zombi pea and cowpea, to transfer the former's genetic resistance genes to pests and diseases, has been reported\textsuperscript{17}.

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Bruchids or seed weevils (Coleoptera: Bruchidae) are a group of insects that destroy the seeds of legume crops. Bruchid infestations of Vigna crops initially occur in fields where female bruchids lay eggs on young pods. The larvae bore through the pods to the seeds. Here, the larvae grow and develop into adults by consuming the seed’s nutrients. After harvest, the adult bruchids emerge from the seeds and start new infestations by laying eggs directly on stored seeds, which can result in a total loss of a seed lot within 2–4 months. Although chemical fumigation can be used to control bruchid infestations, the use of resistant cultivar(s) is the best management strategy. In general, seeds of cultivated Vigna species are susceptible to bruchids, especially the cowpea weevil (C. maculatus L.) and azuki bean weevil (C. chinensis F.). These pests are widely distributed in nearly all continents owing to international seed trading. However, in general, seeds of both cultivated and wild V. vexillata are completely resistant to the C. maculatus and Z. subfasciatus, and this resistance results from the presence of the non-protein amino acid para-aminophenylalanine (PAPA) in the seeds.

Although V. vexillata has the potential to be a source of resistance genes to biotic and abiotic stresses for plant breeding and to be a new feed crop in the future, little is known of its genetics and limited genomic resources are available. Therefore, it is necessary to increase our knowledge and develop genomic tools to exploit the genetic potential of V. vexillata for use in breeding Vigna and other crops. Here, our objectives were to (i) develop a high-density linkage map for V. vexillata and (ii) identify quantitative trait loci (QTL) for seed resistance to C. chinensis and C. maculatus in V. vexillata.

Results
Specific Locus Amplified Fragment Sequencing (SLAF-seq) data and genotyping. After pre-processing, 187.10 Gb of raw data, containing 733.52 M reads, were generated. On average, the Q30 (quality scores of at least 30, indicating a 1% chance of error, and thus 99% confidence) was 94.05%, and the GC content was 36.63%. The numbers of reads for cultivated zombi pea ‘TVNu240’ (resistant) and wild zombi pea ‘TVNu 1623’ (susceptible) were 23,183,922 and 24,285,995, respectively. The total read number for offspring was 3,464,884. After read clustering, 4,044,822 single nucleotide polymorphisms (SNPs) were detected of which 2,202,256 (55.45%) were polymorphic. After filtering the SNP markers lacking the parent information and showing low read depths, 378,433 polymorphic markers were successfully genotyped and grouped into eight segregation patterns, ab × cd, ef × eg, hk × hk, lm × ll, nn × np, aa × bb, ab × cc, and cc × ab. In total, there were 233,975 SNP markers showing the segregation pattern of aa × bb.

Characteristics of the genetic map. After a series of screenings, 6,954 SNP markers were used for a genetic linkage analysis. Among these markers, 6,529 (93.89%) were successfully clustered into a linkage map of 11 linkage groups (LGs) (Fig. 1), indicating the relatively high quality of the genetic map. Table 1 summarises the characteristics of the linkage map constructed in this study. The map was 1,740.8 cM in length, with an average distance of 0.27 cM between adjacent markers. The numbers of markers per LG ranged from 267 on LG10 to 1,080 on LG3, with an average of 593.5 markers. The shortest LG was 108.52 cM (LG10), and the longest LG was 193.13 cM (LG3) (Table 1; Fig. 1). The average length of a LG was 158.25 cM. Large gap(s) (gap between two adjacent markers >10 cM) existed on LGs 2, 3, 5, 6, and 10.

Comparative genome analysis. The genome comparison revealed the presence of highly conserved microsyntenic blocks between zombi pea and cowpea, mungbean, azuki bean, and common bean (Fig. 2; Supplementary Table S1). The zombi pea genome showed the greatest synteny with the cowpea genome. Zombi pea LGs 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 corresponded to cowpea chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11, respectively, to mungbean chromosomes 2/3/5, 11, 7, 1, 4/5, 10, 8, 6, 5, 9, and 2, respectively; to azuki bean chromosomes 4/7, 10, 1, 11, 4/7, 5, 8, 3, 2, 9, and 6, respectively, and to common bean chromosomes 1/5, 2/3, 2/3, 4, 5/8, 6, 7, 1/8, 9, 10, and 11, respectively.
| Linkage group | No. of Markers | Length (cM) | Average distance between markers (cM) | Maximum gap (cM) |
|---------------|---------------|-------------|---------------------------------------|------------------|
| 1             | 546           | 151.6       | 0.28                                  | 7.58             |
| 2             | 494           | 157.4       | 0.32                                  | 14.84            |
| 3             | 1,080         | 193.1       | 0.18                                  | 11.81            |
| 4             | 643           | 167.3       | 0.26                                  | 3.05             |
| 5             | 440           | 156.7       | 0.36                                  | 13.74            |
| 6             | 470           | 136.3       | 0.29                                  | 16.03            |
| 7             | 773           | 188.3       | 0.24                                  | 2.43             |
| 8             | 517           | 168.7       | 0.33                                  | 3.09             |
| 9             | 882           | 184.4       | 0.21                                  | 2.32             |
| 10            | 267           | 108.5       | 0.41                                  | 17.88            |
| 11            | 417           | 128.5       | 0.31                                  | 7.52             |
| Total         | 6,529         | 1,740.8     | 0.27                                  | 17.88            |

Table 1. Features of the high-density genetic linkage map constructed for the Vigna vexillata F₂ population of a cross between ‘TVNu 240’ and ‘TVNu1623’ using SNP markers generated by SLAF sequencing.

Figure 2. Circos map showing genome synteny between the genetic linkage map of Vigna vexillata (LG01–11) and the reference genome sequences of cowpea (V. unguiculata; Vu01–11), mungbean (V. radiata; Vr01–11), azuki bean (V. angularis; Va01–11), and common bean (Phaseolus vulgaris; Pvu01–11).
Variation in resistance to *C. maculatus* and *C. chinensis* in the parents and F2 population. The zombi pea varieties TVNu 240 and TVNu 1623 differed in their responses to *C. chinensis* infestation, with 0% and 48.67%, respectively, of seeds being damaged. The area under the disease progress stairs (AUDPS), which indicates the progression of infestation severity, was 0 in TVNu 240 and 705 in TVNu 1623. In the F2 population the level of damage caused by *C. chinensis* varied from 0% to 96%, with an average of 11.60%, and the AUDPS varied from 0 to 1,970, with an average of 190.61.

In addition, TVNu 240 and TVNu 1623 had very different responses to *C. maculatus* infestation, with 0% and 94%, respectively, of seeds being damaged. The AUDPS was 0 in TVNu 240 and was 2,115 in TVNu 1623. In the F2 population, the level of damage caused by the bruchids varied from 0% to 98%, with an average of 26.99%, and the AUDPS varied from 0 to 2,193.88, with an average of 552.52.

The frequency distributions of percentage of seeds damaged and the AUDPS as a result of *C. chinensis* and *C. maculatus* infestations in the F2 population were continuous but highly skewed towards the resistant TVNu 240 (Fig. 3a,b). Thus, resistance to *C. chinensis* and *C. maculatus* in TVNu 240 appears to be a quantitative trait and may be controlled by several genes.

Heritability. Broad-sense heritability ($H^2$) estimates for the percentage of seeds damaged by *C. chinensis* and *C. maculatus* were 57.55% and 98.44%, respectively. The $H^2$ estimates for AUDPS resulting from *C. chinensis* and *C. maculatus* infestations were 87.70% and 98.24, respectively. These indicated that resistance to *C. chinensis* and *C. maculatus* was principally controlled by genetic factors.

QTL analysis. For *C. chinensis*, three (one major and two minor) QTLs on LGs 3, 6, and 11 were identified for percentage of damaged seeds (Table 2; Fig. 4). The major QTL, $qCc_{PDS6.1}$, was at 78 cM, between Marker79444 and Marker79577, on LG6 and explained 25.51% of the total trait variation (PVE) in the F2 population. The two minor QTLs each accounted for less than 10% of PVE in the population. At these QTLs, alleles from TVNu 240 decreased the percentage of damaged seeds. One major and three minor QTLs each on LGs 2, 3, 6, and 11 were identified for AUDPS (Table 2; Fig. 4). The major QTL, $qCc_{AUDPS6.1}$, was at 78 cM on LG6 and explained 23.99% of the PVE. All the minor QTLs showed PVE values less than 10%. At all the QTLs detected for AUDPS, alleles from TVNu 240 increased AUDPS (increased the resistance). The QTLs for the percentage of damaged seeds and AUDPS identified on the same LG were located in the same region (Table 2; Fig. 4).

In case of *C. maculatus*, one major and one minor QTLs on LGs 6 and 11 were found for the percentage of damaged seeds (Table 2; Fig. 4). The major QTL, $qCm_{PDS6.1}$, was at 81 cM, between Marker197124 and Marker196422, on LG6 and explained 52.41% of the PVE. The minor QTL, $qCm_{PDS11.1}$, was located at 125 cM, between Marker375983 and Marker376183, and explained 7.21% of the PVE. At these two QTLs, alleles from


TVNu 240 decreased the percentage of damaged seeds. Similarly, one major and one minor QTLs on LGs 2
and 11 were identified for AUDPS (Table 2; Fig. 4). The major QTL, qCm_AUDPS6.1, was at the same position
as qGc_AUDPS6.1 and explained 52.46% of the PVE. The minor QTL, qCm_AUDPS11.1, was at 67 cM, between
Marker360510 and Marker360570, on LG11 and explained 6.26% of the PVE. At both qCm_AUDPS6.1 and qCm_-
AUDPS11.1, alleles from TVNu 240 increased the AUDPS. The major QTLs for resistance to C. maculatus and C.
chinensis were located in the same genomic region.

**Discussion**

In recent years, underutilised legume crops, including zombi pea, have been praised as potential sources of genes
for crop improvement and food security in the face of climate change and future population increases31. Zombi
pea is resistant to several biotic and abiotic stresses. Gene discovery in zombi pea can be useful for crop improve-
ment. However, the lack of genomic resources and breeding tools in zombi pea hinders gene discovery in this
species. Next-generation sequencing technologies are highly useful for genomics study of underutilised crops33.
Genetic linkage mapping is important in genomics studies aimed at crop improvement because they facilitate
gene mapping and association analyses needed for gene discovery22. In this study, we successfully constructed
a high-density linkage map for zombi pea that will be used to identify genomic regions associated with bruchid
resistance.

**Linkage map of zombi pea.** Previously, there were only three linkage maps for zombi pea, with the first
linkage map of this species being developed 13 years ago22. The map developed by Marubodee et al.24 had
the greatest number of markers and density. It was composed of 559 markers (84 simple sequence repeats and
475 SNPs based on restriction site-associated DNA-tagged sequences) markers, with a mean distance of 1.8 cM
between adjacent markers, which grouped into 11 LGs. In our present study, by applying the SLAF-seq tech-
nique and the reference genome sequence of cowpea, the linkage map developed for zombi pea was substantially
improved, containing 6,529 SNP markers that resolved into 11 LGs, with a mean distance of 0.27 cM between
adjacent markers. Compared with the high-density linkage maps developed for mungbean26, azuki bean27, and
cowpea28, our linkage map contained a greater number of markers than the mungbean (1,321 markers) and azuki
bean (2,032 markers) maps but less than the cowpea map (17,739 markers). The high-density linkage map devel-
oped in this study is useful for genomics studies of zombi pea.

**Genome synteny with related legume species.** Previous comparative genome analyses among species
belonging to the genus Vigna, including zombi pea, cowpea, mungbean, and azuki bean, based on common
markers of linkage maps have revealed extensive macro-syntenic relationships34,35. In this study, the syntenic
maps based on sequence comparisons revealed highly extensive micro-synteny levels among zombi pea and other
Vigna species, as well as common bean. All these species have the same number of chromosomes (2n = 2x = 22).
Previous studies showed high genome conservation between Vigna species and common bean28,31. As expected,
a synteny analysis revealed that zombi pea had the greatest level of genome conservation with cowpea compared
with other legume species. Zombi pea and cowpea both originated from Africa and share several common mor-
phological characteristics32. Although zombi pea and cowpea are classified into different subgenera (Plectrotropis
and Vigna, respectively) of the genus Vigna, successful hybridization between the two species has been reported18.
Owing to the high level of genome conservation (Fig. 2), available reference genome sequences of cowpea30 can
be used for genomic analysis and gene identification in zombi pea.

**Genetics of bruchid resistance in zombi pea.** In general, zombi pea is completely resistant to C. macu-
latius14,23; therefore, it is not possible to investigate the genetics of the resistance. To investigate the genetics of the
bruchid resistance in zombi pea, a large set of zombi pea germplasm (>400 accessions) from several countries

### Table 2.  QTLs associated with seed resistance to *Callosobruchus chinensis* and *Callosobruchus maculatus*
detected in the F2 population of the cross between cultivated zombi pea ‘TVNu240’ and wild zombi pea
‘TVNu1623’. *PDS* = percentage of damaged seeds, *AUDPS* = area under the disease progress stair. *a* linkage
group. *b* Position (centimorgan) on the linkage map. *c* Phenotypic variance explained by the QTL. *d*
Additive effect. *e* Dominant effect.

| Bruchid species | Trait | LG | QTL name | Position | LOD score | PVE (%) | Add | Dom |
|----------------|-------|----|----------|----------|-----------|---------|-----|-----|
| C. chinensis   | PDS   | 3  | qGc_AUDPS3.1 | 65.4 | 4.80 | 8.33 | 0.15 | -9.38 |
|                |       | 6  | qGc_AUDPS6.1 | 78.0 | 14.12 | 27.14 | 11.72 | -0.94 |
|                |       | 11 | qGc_AUDPS11.1 | 99.6 | 4.60 | 7.86 | 6.17 | -3.44 |
|                | AUDPS | 2  | qGc_AUDPS2.1 | 6.0  | 4.75 | 7.65 | 82.45 | -105.17 |
|                |       | 3  | qGc_AUDPS3.1 | 66.8 | 4.94 | 8.42 | 14.79 | -161.10 |
|                |       | 6  | qGc_AUDPS6.1 | 78.0 | 12.77 | 23.76 | 191.50 | -1.26 |
|                |       | 11 | qGc_AUDPS11.1 | 99.6 | 4.86 | 8.15 | 107.99 | -65.41 |
| C. maculatus   | PDS   | 6  | qGm_AUDPS6.1 | 80.6 | 33.28 | 52.41 | 27.33 | -20.26 |
|                |       | 11 | qGm_AUDPS11.1 | 125.6 | 6.61 | 7.21 | 11.38 | -6.70 |
|                | AUDPS | 6  | qGm_AUDPS6.1 | 80.0 | 30.74 | 51.82 | 444.63 | -430.01 |
|                |       | 11 | qGm_AUDPS11.1 | 128.4 | 4.84 | 5.66 | 195.12 | -156.34 |
were screened against *C. maculatus* and *C. chinensis* (P. Somta, unpublished data) to identify susceptible zombi pea varieties. Most of the germplasms were completely resistant to both bruchid species, but some accessions were moderately or highly susceptible to the bruchids. As a result, TVNu 240 and TVNu 1623 were used as resistant and susceptible parents, respectively, in this study.

A continuous distribution but with high levels of skewedness towards the resistant parent for the percentage of damaged seeds, resulting from both *C. chinensis* and *C. maculatus* infestations, were observed in the F₂ population (Fig. 3a,b). This suggested that the resistance is quantitative, and may be controlled by one or a few major locus/loci with dominance over the susceptible loci coupled with some modifying loci. In *Vigna* species, both quantitative and qualitative loci control resistance to *C. chinensis* and/or *C. maculatus*. The quantitative resistance against bruchids has been reported in rice bean⁴⁻⁵, black gram⁶, and *Vigna vexillata*⁷. Nonetheless, the high heritability estimated for the bruchid resistance (~90%) in *V. vexillata* suggests that the resistance in this species is mainly the result of genetic factor(s).

**QTLs for bruchid resistance in zombi pea.** The QTL analysis identified one major and three minor QTLs for *C. chinensis* resistance and one major and two minor QTLs for *C. maculatus* resistance in zombi pea accession TVNu 240 (Table 2; Fig. 4). Among these QTLs, the major QTLs *qCc_PDS6.1* and *qCc_AUDPS6.1* (that conferred resistance to *C. chinensis*), as well as the major QTLs *qCc_PDS6.1* and *qCc_AUDPS6.1* (that conferred resistance to *C. maculatus*), were mapped to a similar position. These QTLs are very likely the same locus. Thus, we considered them the same QTL, named *qBr6.1*. Similarly, the minor QTLs *qCc_PDS11.1* and *qCc_AUDPS11.1* that conferred resistance to *C. chinensis* were mapped to the same position. Thus, we considered them the same QTL, named *qBr11.1*. In addition, the minor QTLs *qCm_PDS11.1* and *qCm_AUDPS11.1* that conferred resistance to *C. maculatus* were mapped to a similar position. Again, they were considered the same QTL, named *qBr11.2*. *qBr11.1* and *qBr11.2* were ~30 cM apart.

Among *Vigna* species, bruchid resistance has been well studied in mungbean⁸⁻¹¹, in which a major QTL, *qBr5.1*, for resistance to *C. chinensis* and *C. maculatus*, is located on chromosome 5 and a gene encoding a polygalacturonase inhibitor is a candidate resistance gene. The markers associated with the *qBr6.1* for bruchid resistance in zombi pea, Marker197124 and Marker196422, are on mungbean chromosome 10. Therefore, *qBr6.1* in zombi pea and *qBr5.1* in mungbean are not the same locus.

Although Birch *et al.*¹² reported that PAPA in seeds of zombi pea acts as a defence chemical against *C. maculatus* and *Z. subfasciatus*, Bresson⁴³ showed that some zombi pea accessions, differing in resistance to *C. maculatus*, do not have significantly different amounts of PAPA in their mature seeds. This suggested that PAPA may not be the principal defence chemical against *C. maculatus* and *Z. subfasciatus* in zombi pea. Lattanzio *et al.*¹⁴ demonstrated that, in general, seeds of zombi pea resistant to *C. maculatus* contain high contents of an α-amylase inhibitor. The α-amylase inhibitor from common bean is an antibiosis that negatively affects the growth and development of *C. chinensis*, *C. maculatus*, and *Z. subfasciatus*⁶,⁶. Genome comparisons revealed that
Marker197124 and Marker196422 flanking qBr6.1, which confers resistance to C. chinensis and C. maculatus in zomo pea, were on common bean chromosome 6 at the positions 18.726 Mb and 19.329 Mb, respectively. Three genes Phvul.006G087700 (Chr06: 19,974,245–19,976,281), Phvul.006G200500 (Chr06: 29,738,762–29,740,823), and Phvul.006G200880 (Chr06: 29,750,251–29,752,428), each coding for α-amylase inhibitors, are on common bean chromosome 6. Phvul.006G087700 is ~650 Kb away from Marker196422 and can be considered a candidate gene for bruchid resistance in zomo pea. Further studies are necessary to confirm the association between this gene and resistance in zomo pea.

Materials and Methods

Plant materials. An F2 population of 198 individuals derived from the cross ‘TVNu 240’ × ‘TVNu 1623’ was used in this study. TVNu 240 is a cultivated zomo pea (V. vexillata var. macroserma) from the Central African Republic and is completely resistant to C. maculatus and C. chinensis, while TVNu 1623 is a wild zomo pea from Nigeria and is highly susceptible to C. maculatus and moderately susceptible to C. chinensis. The seeds of these two accessions were obtained from the International Institute of Tropical Agriculture, Ibadan, Oyo State, Nigeria.

The F2 population and 10 plants of each parent were grown under field conditions using 0.75 × 0.75 m spacing at Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand from December 2017 to March 2018. Genomic DNA was extracted from young leaves of each F2 and parental plant using the CTAB method[47]. Mature pods and seeds of each plant were harvested separately for bruchid evaluation.

SLAF-seq marker analysis. The genomic DNAs extracted from the parental lines and from each of the 198 F2 plants were used for SLAF library construction and sequencing following Sun et al.[48], with minor modifications. Briefly, a pre-experiment SLAF-seq analysis was performed using the reference genome of cowpea[29] to obtain the optimal restriction enzymes, which would avoid repetitive SLAFs, and an even SLAF distribution. Then, a SLAF library was prepared based on the results of the pre-experiment analysis. Genomic DNA from each entry was digested at 37 °C with HinCII and Rsal (NEB, Ipswich, MA, USA), incubated with the Klnone Fragment (3′ → 5′exonuclease) (NEB) and dATP at 37 °C to add single-nucleotide A overhangs to the digested fragments, and the A-tailed DNA fragments were then ligated to Duplex Tag-labelled Sequencing adapters (PAGE purified, Life Technologies) using T4 DNA ligase. PCR was performed using diluted restriction-igation DNA samples, dNTPs, Q5 High-Fidelity DNA Polymerase and PCR primers (Forward primer: 5′-AATGATACGGGACACCAGCA-3′, reverse primer: 5′-CAAGCAGAAGACGGCATACG-3′) (PAGE-purified, Life Technologies). PCR products were then purified using Agencourt AMPure XP beads (Beckman Coulter, High Wycombe, UK) and pooled. Pooled samples were separated using 2% agarose gel electrophoresis. Fragments ranging from sizes 314 to 464 bp (with indexes and adaptors) were excised and purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). Gel-purified products were diluted. Paired-end sequencing (each end being 125 bp) was performed using an Illumina HiSeq X-ten system (Illumina, Inc; San Diego, CA, USA) in accordance with the manufacturer’s recommendations at the Biomarker Technologies Corporation (Beijing, China).

Sequence data grouping and genotyping. Reads generated from the sequencing were compared to the cowpea reference genome sequence using BWA software. SLAF marker identification and genotyping were performed as per by Sun et al.[48]. Low-quality reads (quality score <20e) were eliminated and the raw reads were assigned to 198 F2 individual samples using the duplex barcode sequences. After trimming the barcodes and the terminal 5-bp positions from each high-quality read, the clean reads were clustered together based on their sequence identities. Sequences that mapped to the same locus with over 90% identity levels were defined as one SLAF locus. SNP loci between the two parents were detected in which SLAFs with >3 SNPs were removed. Alleles of each SLAF were defined based on parental reads with sequence depths >10-fold and offspring reads with sequence depths >2-fold. In a diploid species, such as zomo pea, one marker locus can contain a maximum of four genotypes; thus, SLAF loci with >4 alleles were discarded. Nonetheless, only SLAFs with 2, 3, or 4 alleles were considered potential markers. Because V. vexillata is a self-pollinating species, only polymorphic markers showing homozygous states between the parents were selected. Genotype scoring was conducted using a Bayesian approach as described by Sun et al.[48]. Subsequently, three processes were performed to screen for high-quality markers. First, markers with average sequence depths less than fourfold in the parents were discarded. Second, markers having >25% missing data were removed. Third, markers with significant segregation distortion (P < 0.001) were initially excluded from genetic map construction and then added later as accessory markers.

Genetic map construction. Marker loci were partitioned primarily into LGs based on their locations on the cowpea reference genome. Next, the modified logarithm of odds (MLOD) scores between markers was calculated to further confirm the robustness of the markers for each LG. Markers with MLOD scores < 5 were filtered prior to ordering. HighMap strategy[39] was used to order the SNP markers and correct genotyping errors within LGs. First, recombinant frequencies and LOD scores were calculated using a two-point analysis to infer linkage phases. Then, enhanced Gibbs sampling, spatial sampling and simulated annealing algorithms were combined to conduct the iterative process of marker ordering. The error correction strategy of SMOOTH[38] was used according to parental contributions to genotypes, and a k-nearest neighbour algorithm was applied to impute missing genotypes. Skewed markers were added to this map by applying a multipoint method of maximum likelihood. Map distances were estimated using Kosambi’s mapping function.

Genome synteny analysis. To determine genome conservation between zomo pea and other closely related legumes, the SNP markers used to construct the linkage map were subjected to a BLASTN search against reference genome sequences of cowpea [V. unguiculata (L) Walp.][39], mungbean [V. radiata (L) Wilczek][39], azuki bean [V. angularis (Ohwi) Ohwi and Ohashi][39], and common bean [P. vulgaris L.][39]. The synteny levels between...
Evaluation of seed resistance to *C. maculatus* and *C. chinensis*. A population of *C. chinensis* and a population of *C. maculatus* were reared on seeds of susceptible mungbean cultivar 'Kamphaeng Saen 2' and under controlled conditions in a room at 28 °C and 60% relative humidity. The evaluations of resistance to these insects were carried out following the procedures described by Somta et al., with minor modifications. Briefly, 40 seeds from each plant in the F₂ population were placed in a plastic box. Then, 10 pairs (10 males and 10 females) of newly emerged adult insects were introduced into the box, allowed to lay eggs for 7 days and then removed. The infested seeds were maintained at 28 °C and 60% relative humidity. The numbers of seeds damaged by the bruchids [seeds with hole(s)] were counted at 30 days after insect introduction (DAI), and then counted every 5 days until 60 DAI. Each time, damaged seeds were removed from the boxes. The cumulative number of total seeds damaged by the bruchids at each counting dates was calculated and converted into percentage. Then, the percentage of damaged seeds was used to calculate the AUDPS. AUDPS is an improve version of area under disease progress curve, which measures the progressive development of disease severity in plants. Thus, AUDPS is an indicator of the progression of the bruchid infestation severity in this study. The percentage of damaged seeds at 60 DAI and the AUDPS value of each F₂ plants were used for data analyses.

Heritability estimation for the resistance. The $H^2$ values of PDS and AUDPS caused by each bruchid species in the F₂ population were calculated using following formula:

$$H^2 = \frac{\sigma^2_{F2} - \left( \frac{\sigma^2_{TVNuv240X} + \sigma^2_{TVNuv1623Y}}{2} \right)}{\sigma^2_{F2}},$$

where $\sigma^2_{F2}$, $\sigma^2_{TVNuv240X}$, and $\sigma^2_{TVNuv1623Y}$ are variances of the F₂ population, TVNu 240, and TVNu 1623, respectively.

QTL analysis. The QTL analysis was conducted using inclusive composite interval mapping (ICIM) method implemented in QTL IciMapping 4.1 software. The percentage of damaged seeds and AUDPS resulting from *C. chinensis* and *C. maculatus* infestations were used to locate QTLs associated with pest resistance. The ICIM was performed at every 0.1 CM using a probability in stepwise regression of 0.001. A significant LOD threshold for QTLs of each trait was determined using a 3,000 permutation test at $P = 0.01$.

References
1. Tomooka, N. et al. *Vigna* species. In *Broadening the Genetic Base of Grain Legumes* (eds Singh, M. et al.) 175–208. (Springer, 2014).
2. Tomooka et al. *Vigna* Genetic Resources. In: The 14th NIAS International Workshop on Genetic Resources: Genetic Resources and Comparative Genomics of Legumes (Glycine and Vigna) (eds Tomooka, N. & Vaughan, D. A.) 11–21 (National Institute of Agrobiological Sciences, Japan, 2010).
3. Duke, J. A. *Vigna unguiculata* (L.) Walp. spp. *unguiculata*. In *Legumes of World Economic Importance* (ed. Okeson, O. N.) 303–305 (Plenum Press, 1981).
4. Lawin, R. J. & Cottrell, A. Wild mungbean and its relatives in Australia. *Biologist* 35, 267–273 (1988).
5. Sasikumar, B. & Sardana, S. *Vigna vexillata* (Fabaceae), a pulse cum tuber crop of northeastern hill region of India. *Econ. Bot.* 42, 292 (1988).
6. Karunawadan, A. et al. *Vigna vexillata* (L.) A. Rich. cultivated as a root crop in Bali and Timor. *Genet. Resour. Crop Evol.* 53, 213–217 (2006).
7. Chandel, K. P. S. et al. *Vigna capensis* Walp. (V. vexillata) an edible root legume. *Curr. Sci.* 41, 537 (1972).
8. Bhattacharyya, P. K. et al. Grow *Vigna vexillata* for protein-rich tuber-cum-pulse crop in North-eastern hill region. *Seeds Farm* 10, 33–36 (1984).
9. Lawn, R. J. & Watkinson, A. R. Habitat, morphological diversity and distribution of the genus *Vigna* Savi in Australia. *Aust. J. Agric. Res.* 53, 1305–1316 (2002).
10. Vanderborgh, T. Some observations on seedlings of *Vigna vexillata* (L.) A. Rich. (Fabaceae). *Bull. Jard. Bot. Nat. Belg.* 59, 179–187 (1989).
11. Roeklein, J. C. & Leung, P. A profile of economic plants. Transaction Publishers, USA (1987).
12. Miller, I. L. & Williams, W. T. Tolerance of some tropical legumes to six months simulated waterlogging. *Trop. Grasslands* 51, 39–43 (1980).
13. Birch, A. N. E. et al. Para-aminophenylalanine in *Vigna*: possible taxonomic and ecological significance as a seed defence against bruchids. *Phytochemistry* 25, 2745–2749 (1986).
14. Jackia, L. E. N. & Oghiahi, S. Pod wall richness and resistance of two wild cowpea, *Vigna vexillata*, accessions to *Maruca testulalis* (Geyer) (Lepidoptera: Pyralidae) and *Clavigralla tomentosicollis*, accessions to *Vigna vexillata* *Maruca testulalis* through isolation of *Vigna* subgenus *Ceratotropis*. *Euphytica* 115, 27–41 (2000).
15. Jackia, L. E. N. et al. Resistance to the legume pod borer, *Maruca vitrata* Fabricius, and the probable modalities involved in wild. *Vigna*. *Crop Prot.* 15, 753–761 (1996).
16. Thottappilly, G. et al. Screening germplasm of *Vigna vexillata* for resistance to cowpea mottle virus. *Int. J. Trop. Plant Dis.* 12, 75–80 (1994).
17. Gomathinayagam, P. et al. Interspecific hybridization between *Vigna unguiculata* (L.) Walp. and *V. vexillata* (L.) A. Rich. through in *vitro* embryo culture. *Euphytica* 102, 203–209 (1998).
18. Srinivas, P. et al. Genetics and breeding of resistance to bruchids (*Callosobruchus spp*) in *Vigna* crops: a review. *NU Int. J. Sci.* 4, 1–17 (2007).
19. Tomooka, N. et al. The effectiveness of evaluating wild species: searching for sources of resistance to bruchid beetles in the genus *Vigna* subgenus *Ceratotropis*. *Euphytica* 115, 27–41 (2000).
20. Considine, M. J. et al. Nature’s pulse power: legumes, food security and climate change. *J. Exp. Bot.* 68, 1815–1818 (2017).
21. Collard, B. et al. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica* 142, 169–196 (2005).
22. Ogundiw, E. A. et al. A genetic linkage map of *Vigna vexillata*. *Plant Breed.* 124, 392–398 (2005).
23. Maruboeke, R. et al. Construction of an SSR and RAD-marker based molecular linkage map of Vigna vexillata (L.) A. Rich. PLoS One 10(9), e0138942, https://doi.org/10.1371/journal.pone.0138942 (2015).
24. Dachapak, S. et al. QTL analysis of domestication syndrome in zombi pea (Vigna vexillata), an underutilized legume crop. Preprint at https://biorxiv.org/content/early/2018/06/21/353029 (2018).
25. Kang, Y. J. et al. Genome sequence of mungbean and insights into evolution within Vigna species. Nat. Comm. 5, 5443, https://doi.org/10.1038/ncomms4433 (2014).
26. Liu, C. et al. Development of a high-density genetic linkage map and identification of flowering time QTLs in adzuki bean (Vigna angularis). Sci. Rep. 23, 6,39523, https://doi.org/10.1038/srep39523 (2016).
27. Lo, S. et al. Identification of genetic factors controlling domestication-related traits in cowpea (Vigna unguiculata L. Walp). Sci. Rep. 19(8), 6261, https://doi.org/10.1038/s41598-018-24349-4 (2018).
28. Kang, Y. J. et al. Draft genome sequence of adzuki bean, Vigna angularis. Sci. Rep. 28(5), 8069, https://doi.org/10.1038/srep08069 (2015).
29. Muñoz-Amatriain, M. et al. Genome resources for climate-resilient cowpea, an essential crop for food security. Plant J. 89, 1042–1054 (2017).
30. Yang, K. et al. Genome sequencing of adzuki bean (Vigna angularis) provides insight into high starch and low fat accumulation and domestication. PNAS 112(21), 3213–3218 (2015).
31. Marchal, R. et al. Etude taxonomique d’un groupe d’espèces des genres Phaseolus et Vigna (Papilionaceae) sur la base des données morphologiques et polliniques, traitées pour l’analyse informatique. Boissiera 28, 1–273 (1978).
32. Kitch, L. W. & Shade, R. E. Seed and pod resistance to Callosobruchus maculatus among various Vigna species. Insect Sci. Appl. 14, 333–341 (1993).
33. Somta, P. et al. Development of an interspecific Vigna linkage map between Vigna unghellata (Thunb.) Ohwi & Ohashi and V. nakadhatinae (Ohwi) Ohwi & Ohashi and its use in analysis of bruchid resistance and comparative genomes. Plant Breed. 125, 77–84 (2006).
34. Venkataramana, P. B. et al. Mapping QTL for bruchid resistance in rice bean (Vigna unghellata). Euphytica 207, 135–147 (2016).
35. Souframanien, J. et al. Identification of quantitative trait loci for bruchid (Callosobruchus maculatus) resistance in black gram (Vigna mungo (L.) hepper). Euphytica 176, 349–356 (2010).
36. Somta, P. et al. Mapping of quantitative trait loci for a new source of resistance to bruchids in the wild species Vigna nepalensis Tateishi and Maxted (Vigna subgenus Ceratotropis). Theor. Appl. Genet. 117, 621–627 (2008).
37. Somta, P. et al. Inheritance of seed resistance to bruchids in cultivated mung bean (Vigna radiata L. Wilczek). Euphytica 155, 47–55 (2007).
38. Dongre, T. K. et al. Identification of resistant source to cowpea weevil [Callosobruchus maculatus (F.)] in Vigna sp. and inheritance of their resistance in black gram (Vigna mungo var. mungo). J. Stored Prod. Res. 32, 201–204 (1996).
39. Adjadi, O. et al. Inheritance of bruchid resistance in cowpea. Crop Sci. 25, 740–742 (1985).
40. Chotechung, S. et al. A gene encoding a polygalacturonase-inhibiting protein (PGIP) is a candidate gene for bruchid (Coleoptera: bruchidae) resistance in mungbean (Vigna radiata). Theor. Appl. Genet. 129, 1673–1683 (2016).
41. Kaewwongwal, A. et al. Novel alleles of two tightly linked genes encoding polygalacturonase-inhibiting proteins (VrPGIP1 and VrPGIP2) associated with the Br locus that confer bruchid (Callosobruchus sp.) resistance to mungbean (Vigna radiata) accession V2709. Front. Plant Sci. 28(8), 1692, https://doi.org/10.3389/fpls.2017.01692 (2017).
42. Bressan, R. A. Contributions of PAPA to V. vexillata resistances: another opportunity for biotechnology? In Joint Cowpeas Biotechnology Workshop, 16–20, (Purdue University, 1990).
43. Lattanzio, V. et al. Seed coat tannins and bruchid resistance in stored cowpea seeds. J. Sci. Food Agric. 85, 839–846 (2005).
44. Ishimoto, M. & Kitamura, K. Growth inhibitory effects of an α-amylase inhibitor from the kidney bean, Phaseolus vulgaris (L.) on three species of bruchids (Coleoptera: Bruchidae). Appl. Entomol. Zool. 24, 281–286 (1989).
45. Shade, R. E. et al. Transgenic pea seeds expressing the α-amylase inhibitor of the common bean are resistant to bruchid beetles. Bio/ Techno. 12, 793–796 (1994).
46. Lodhi, M. A. et al. A simple and efficient method for DNA extraction from grapevine cultivars and Vitis species. Plant Mol. Biol. Rep. 12, 6–13 (1994).
47. Sun, X. et al. SLAF-seq: an efficient method of large-scale de novo SNP discovery and genotyping using high-throughput sequencing. PLoS ONE 8, e58700, https://doi.org/10.1371/journal.pone.0058700 (2013).
48. Liu, D. et al. Construction and analysis of high-density linkage map using high-throughput sequencing data. PLoS ONE 9,e98855, https://doi.org/10.1371/journal.pone.0098855 (2014).
49. Van, O. S. et al. SMOOTH: a statistical method for successful removal of genotyping errors from high-density genetic linkage data. Theor. Appl. Genet. 112, 187–194 (2005).
50. Schmutz, J. et al. A reference genome for common bean and genome-wide analysis of dual domestications. Nat. Genet. 46, 707–713 (2014).
51. Krzywinski, M. et al. Circos: An information aesthetic for comparative genomics. Genome Res. 19, 1639–1645 (2009).
52. Simko, I. & Perpho, H. P. The area under the disease progress stairs: calculation, advantage, and application. Phytopathology 102, 381–389 (2012).
53. Li, H. et al. A modified algorithm for the improvement of composite interval mapping. Genetics 175, 361–374 (2007).
54. Meng, L. et al. QTL IciMapping: Integrated software for genetic linkage map construction and quantitative trait locus mapping in biparental populations. Crop J. 3, 269–283 (2015).

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Author Contributions
K.A., L.W. and S.W. carried out experiments. P.S. initiated, designed, and coordinate the research. P.S., L.W. and X.C. and sought research fund. K.A., L.W. and P.S. analyzed data. P.S., K.A. and L.W. wrote the paper.

Additional Information
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