Copy Number Quantification for the Soybean Cyst Nematode Resistance Locus *rhg1* in the Soybean Varieties of Taiwan

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Abstract: Disease resistance is one of the most successful strategies in crop protection. For example, the implementation of PI 88788 type resistance, which contains high copy numbers of *Resistance to Heterodera glycines* 1 (*rhg1*) loci, into the commercial soybean varieties of the United States has significantly reduced the yield losses caused by soybean cyst nematode (SCN, *H. glycines*). Vegetable soybean, or edamame, has become a major exporting agricultural product in Taiwan with an annual revenue over $80 million USD since 2017. Several local varieties have been developed to fulfill the market needs such as the traits of flavor and sweetness. However, it remains unclear if the historical breeding programs ever incorporated *rhg1* resistance into the varieties of Taiwan. This study applied the TaqMan qPCR method to measure the fluorescent signals specific to the *rhg1* locus on the chromosome 18 of soybean, and the ratio of VIC and FAM signals were analyzed to predict the *rhg1* copy number in the 21 soybean varieties of Taiwan. The results indicated the copy number and the single nucleotide polymorphisms of the 21 soybean varieties were identical to the susceptible soybean variety ‘Williams 82’. As importation of soybean will be continuously needed to fulfill the market and because SCN is absent in the soybean fields of Taiwan, lacking *rhg1* resistance in the local soybean varieties may put the edamame industry at risk and early implementation of SCN resistance in the breeding program, alongside the application of quarantine regulations, will be the key to maintain the SCN-free status and to sustain the edamame industry in Taiwan.

Keywords: copy number variation; edamame; *Resistance to Heterodera glycines* 1 (*rhg1*); *Glycine max* (L.) Merr.; soybean cyst nematode (*Heterodera glycines*); Taiwan; vegetable soybean

1. Introduction

Soybean cyst nematode (SCN, *Heterodera glycines*) is the major yield-limiting biotic stress for soybean production worldwide [1]. The earliest report of cyst nematodes in soybean was documented in Japan [2] as *Heterodera schachtii* Schmidt [3], and was not reclassified as *Heterodera glycines* Ichinohe until 1952 [4]. Since then, the obligate endoparasitic plant nematode has been globally distributed in the major soybean-producing countries including the US, Argentina, Brazil, and China [5]. SCN infects soybean roots, leading to the stunting and yellowing of the host. Moreover, SCN infection often increases soybean susceptibility to fungal infection, which synergistically reduces the yield drastically. SCN caused annual yield losses of about 100 million bushels during 2006 to 2014 [6,7] and it is responsible for approximately over $1 billion loss per year in the United States.

The life cycle of SCN is composed of the egg stage, four juvenile stages, and the adult stage. The second stage juvenile (J2) SCN is the infection stage. The J2 juvenile uses its stylet and enzymes to penetrate soybean roots, and it migrates inside soybean roots until finding a proper feeding site close to the vascular cylinder. SCN secretes a variety...
of effectors to induce a specialized multinucleate feeding structure called syncytium [8]. Syncytia supports SCN growth including the sexual dimorphism at the J3 stage and the sexual maturity at the J4 stage. While the female SCN becomes enlarged and exposed on the root surface, male SCN remains reniform and migrates outside of the roots to fertilize the female SCN. After fertilization, the female SCN dies and becomes a cyst with hundreds of eggs inside. The mature and pigmented cyst provides the eggs a shelter from unfavorable environments, and a new life cycle of SCN begins upon encountering plant hosts and suitable environments [9]. The life cycle of SCN can be completed within thirty days if all conditions are optimized [10]. Therefore, SCN may be considered as a polycyclic disease in a single season of soybean production. Reducing the initial population in soils and preventing the spread of SCN to non-infected areas are important aspects of the SCN management. Tillage, removal of root debris in soil, and rotation with non-host crops such as corn (Zea mays L.) may effectively decrease nematode population [11,12]. Chemical management is commonly used for soil fumigation to reduce nematode population in soils [13]. More recently, seed treatments using the succinate dehydrogenase inhibitor (SDHI) fungicide fluopyram have been proposed for effective SCN-control without eliminating beneficial microorganisms in soils [14]. Nonetheless, the most widely applied and effective management strategy relies on the host resistance.

One of the well-known examples is the introduction of rhg1 (resistance to Heterodera glycines) locus from a resistant donor germplasm line, the plant introduction (PI) 88788, into soybean varieties such as ‘Fayette’ and about 90% commercial soybean varieties over decades [15]. The rhg1 locus has been mapped on the chromosome 18 [16], and the rhg1 haplotype of PI 88788 was defined as rhg1-b [15]. The resistance mechanism of rhg1-b has been shown to depend on the copy number variation, and the resistant PI 88788 contains nine copies of a 31.2 Kb cluster repeat with four candidate genes, including an amino acid transporter (Glyma18g02570, Glyma.18G022400), an α-SNAP (soluble NSF attachment protein) protein (Glyma18g02590, Glyma.18G022500), and a PLAC domain protein with unknown function (Glyma18g02600, Glyma.18G022600), and a wound-inducible protein 12 (Glyma18g02610, Glyma.18G022700). Among these genes, the Glyma.18G022400, Glyma.18G022500, and Glyma.18G022700 have been shown to be involved in SCN resistance by RNA silencing, and their expressions were up-regulated when PI 88788 is affected by SCN [15]. On the other hand, the susceptible variety ‘Williams 82’ contains only one copy of rhg1-c [15,17], and therefore the high copy number in PI 88788 may provide SCN resistance via enhanced expression of these three genes. Among these three genes, only the molecular function of α-SNAP protein in SCN resistance has been clearly studied. It has been shown that rhg1-b type of α-SNAP protein (α-SNAP<sub>Rhg1</sub>HC) has amino acid polymorphism different from the ‘Williams 82’ rhg1-c type of α-SNAP protein (α-SNAP<sub>Rhg1</sub>WT). The α-SNAP<sub>Rhg1</sub>HC exhibited cytotoxicity and low affinity to the N-ethylmaleimide-sensitive factor (NSF), which lead to the disruption of SNARE-dependent vesicle trafficking [18]. The over-expression and hyper-accumulation of α-SNAP<sub>Rhg1</sub>HC may disable the sustainability of feeding cells, which leads to syncytia death and SCN resistance. In addition, α-SNAP protein was shown to interact with two syntaxins, which were initially found in the QTL-mapping studies [19,20] and recently confirmed to be important for SCN resistance [21].

Different from the rhg1-b of PI 88788, the rhg1-a haplotype contains 2.3 to 3 copies in the resistant variety ‘Peking’, and the α-SNAP protein derived from ‘Peking’ represents the low copy version (α-SNAP<sub>Rhg1</sub>LC) [15,17]. Nonetheless, both α-SNAP<sub>Rhg1</sub>HC and α-SNAP<sub>Rhg1</sub>LC were expressed in syncytia and both exhibited cytotoxicity in high dosage [18]. More intriguingly, both types displayed a higher affinity to the NSF on chromosome 7 (NSF<sub>RAN07</sub>, Glyma.07G195900), which contains five amino acid polymorphisms derived from a resistant rhg1-a type variety ‘Forrest’. Their interaction may also explain the segregation disorder observed for the rhg1 on the chromosome 18 and NSF on the chromosome 7 [22]. Other than copy number difference between rhg1-b and rhg1-a type resistant varieties, a retrotransposon (RAC, Rhg1 α-SNAP copia) was found to insert only
in α-SNAP_Rhg1LC, but not α-SNAP_Rhg1HC or α-SNAP_Rhg1WT [23]. It is suggested that RAC has promoter activities, and it is possible that RAC provides additional regulation for rhg1-a.

Other than the rhg1 locus, Rhg4 is another reproducible locus from several mapping studies, and the Rhg4 resistance mostly co-occurs with the rhg1-a type. There are three allelic types of Rhg4, which includes the resistant 'Peking' type Rhg4-a, the susceptible 'Williams 82' type Rhg4-b, and the resistant PI 437654 type Rhg4-c. Copy number variation has also been found for the Rhg4 locus, and 'Peking' was found to have 2.3 copies of Rhg4-a while PI 437654 was found to have 4.3 copies of Rhg4-c [17]. Moreover, the sequence polymorphisms in the promoter regions of resistant types rhg1 and Rhg4 loci were found to regulate higher gene expression that together leads to SCN resistance [17]. Map-based cloning has been applied on the Rhg4 locus, which identified a serine hydroxymethyltransferase (SHMT) with amino acid polymorphisms between the resistant 'Forrest' and the susceptible 'Essex'. The amino acid difference in SHMT may interfere its function in carbon folate metabolism, which leads to hypersensitive response-like cell death in syncytia and provides SCN resistance [24].

Studies on the nature of rhg1 and Rhg4 have provide foundation to develop molecular markers to assist breeding programs. While a competitive allele-specific PCR (KASP) assay has been used to detect amino acid polymorphisms in the α-SNAP_Rhg1HC, α-SNAP_Rhg1LC, and SHMT [25,26], a TaqMan-based quantitative polymerase chain reaction (qPCR) was developed to assess not only the allelic difference, but also the copy number variation [27]. Using the homeolog of Glyma.18G022600 on chromosome 11 (Glyma.11G234400) and the 'Williams 82' genome as a single copy reference, Lee et al. categorized the rhg1 locus into three subtypes, including the F subtype represented by the PI 88788 rhg1-b with nine copies, the P subtype represented by the 'Peking' rhg1-a with three copies, and the W subtype represented by the 'Williams 82' rhg1-c with one copy [27]. By estimating the qPCR fluorescence ratio, the qPCR assay has been applied to estimate many USDA soybean germplasms for their Rhg1 copy number, such as the PI 548402 ('Peking') has 2.8 ± 0.05, the PI 79609 has 7.4 ± 1.57, the PI 88788 was estimated at 9.4 ± 0.78, and the highest in record PI 209332 ('Fayette') has 9.8 ± 0.47 copies [28]. As the Taqman qPCR method has shown its merit in the USDA germplasm, the application to soybean from different regions or countries would extend the understanding of the rhg1 copy number variation in the global soybean varieties.

Soybean production in Taiwan locates mostly in the tropical regions and sub-tropical regions, and several endemic soybean varieties have been bred to improve agronomic traits suitable for the climate of Taiwan. The majority of soybean produced in Taiwan is consumed as vegetable soybean, or edamame, which is a classic appetizer in Japanese restaurants. Edamame produced in Taiwan is mostly exported to Japan, United States, and Canada with over $80 million dollars annually since 2017. However, a comprehensive examination on the SCN risk is lacking, and therefore, this study aims to apply the TaqMan qPCR to document the copy number variation and allelic types of rhg1 in the soybean varieties of Taiwan. The genetic insights will contribute to the SCN risk assessment of the potential disease outbreak.

2. Materials and Methods
2.1. Plant Materials and DNA Extraction

A total of 21 soybean (Glycine max (L.) Merr.) varieties were used in this study (Table 1). Soybean seeds were stored in 4 °C with desiccants to maintain the seed vigor. Soybean seeds were germinated on wet and sterilized tissue papers for five days at room temperature, and the taproots were spliced into a 2 mL screw-cap tube. Each biological replicate (screw-cap tube) contained taproot tissues collected from five soybean individuals. Each soybean variety was represented by four independent biological replicates; in other words, the genotype of each soybean variety was averaged from and represented by 20 soybean individuals. The screw-cap tubes were frozen in −20 °C freezer prior to DNA extraction.
Table 1. Twenty-one soybean varieties of Taiwan tested in this study.

| Variety           | Developer * | Year * | Pedigree *       |
|-------------------|-------------|--------|------------------|
| Hualien No.1 (HL1) | CW Tsai     | 1979   | E32 × Wakajima   |
| Hualien No.2 (HL2) | FH Lin, PH Jan, and CS Chang | 1988 | Dalian-Tou × Wakajima |
| Tainan Select No.1 (TNS1) | CH Wu, TJ Lien, and SS Wang | 2003 | Superior single plant selection from the Cha-Tou variety Edamame |
| Tainan No.3 (TN3)  | TJ Lien, TJ Yu, CH Wu, CS Wu, and YC Wang | 1998 | Local variety of Pingtung black soybean (♀) × HB (♂) |
| Tainan No.4 (TN4)  | TJ Lien, CH Wu, SN Huang, TJ Yiu, and YC Wang | 1999 | Superior single plant selection from local variety Chin-Pi-Tou |
| Tainan No.5 (TN5)  | TJ Lien, TJ Yu, and CH Wu | 1999 | Local variety of Chaohou black soybean (♂) × Tainan No.2(♀) |
| Tainan No.10 (TN10) | CH Wu and HL Huang | 2014 | 95-(1)-4 (Ryokukou × Tainan No.8) (♀) × Tainan No.3 (♂) |
| Tainan No.11 (TN11)| CH Wu       | 2017   | Superior single plant selection from Ryokukou |
| Kaohsiung No.5 (KS5) | KF Chen and SC Cheng | 1995 | Superior single plant selection from Kaohsiung No.2(♂) × Kaohsiung No.5(♀) |
| Kaohsiung No.7 (KS7) | KL Chou and SC Cheng | 2002 | Kaohsiung Select No.1 (♂) × Tanbaguro (♀) |
| Kaohsiung No.9 (KS9) | KL Chou | 2006 | Kaohsiung No.2 (♂) × Kaohsiung No.5 (♀) |
| Kaohsiung Select No.10 (KSS10) | - | 1985 | SRF400 (♂) × SS (♀) |
| Kaohsiung No.11 (KSI11) | KL Chou | 2011 | KVS1329 (♂) × HC (♀) |
| Kaohsiung No.12 (KSI12) | KL Chou | 2012 | Kaohsiung No.6 (♂) × Sayamusume (♀) |
| Hengchun Black Soybean (HBS) | - | - | Germplasm |
| Hei-Wu-Yeh (HWY)   | -           | -      | Germplasm |
| Shih Shih (SS)     | -           | 1957   | Germplasm |
| Hsiang Chi (HC)    | -           | -      | Germplasm |
| Japan Cha-Tou (JCT)| -           | -      | Germplasm |
| Chi-Ren Black Soybean (CRBS) | - | - | Germplasm |
| Chi-Ren-Hei-Long (CRHL) | - | - | Germplasm |

* The “-” in the table indicates untraceable developer, year, or pedigree.

The soybean taproots were mechanically homogenized by the CLUBIO Prep-CB24 homogenizer (Bioman, New Taipei City, Taiwan) at 6.0 speed for 15 s, followed by immediately adding 800 µL GPX1 buffer and 5 µL RNase A of the Genomic DNA Mini Kit (Plant) (Geneaid, New Taipei City, Taiwan). After 10 min of incubation at 60 °C, 200 µL GP2 buffer was added into the mixture and the tube was vortexed and incubated for 3 min on ice. A brief centrifugation of 14,000 rpm for 3 min was performed to precipitate plant tissues debris, and the supernatant was subjected to the subsequent DNA extraction procedure following the manufactural instruction. The final DNA was eluted by distilled deionized water, and the DNA concentration was quantified by the Nanodrop One instrument (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Quantitative Polymerase Chain Reaction (qPCR)

The quantification of rhg1 locus was modified from the methods developed by Lee et al. [27]. The original method applied 12 ng genomic DNA in 5 µL reaction, and the quantification was performed in 384-well plates using the ABI system (Foster City, CA, USA). The modified qPCR condition in this study included 20 ng genomic DNA in 15 µL reaction, and the quantification was performed in 96-well plates using the BioRad CFX Connect qPCR system (Hercules, CA, USA). The sequences of forward primer (5'-GCA GCT GTT GGA ATC ATT CTT TGT T), reverse primer (5'-AAA TTG AAC CTC TTT CTC ATT TGG ATC CT), and the Glyma.18G-specific probe (5'-VIC-BHQ2) as well as the Glyma.11F-specific probe (5'-FAM-BHQ2) followed the design of the original study [27]. The VIC-labeled probe and FAM-labeled probe were synthesized with HPLC cleaning (BIOTOOLS, New Taipei City, Taiwan), and the TOOLS Easy 2X Probe qPCR Mix (BIOTOOLS) was used in this study. Because of the difference in TaqMan qPCR conditions and instruments, the
A probe ratio of FAM to VIC was adjusted to 10:1 to enable the detection of FAM fluorescence signal in soybean varieties PI 88788 and PI 209332, both contain high VIC fluorescence signal that overwhelms the detection of FAM. In other words, the final concentration of both forward primer and reverse primer was 20 μM, and the concentration of FAM-labeled probe and the VIC-labeled probe was adjusted to 0.75 μM and 0.075 μM in each reaction, respectively. Each biological replicate was technically repeated for three times, and the 21 soybean varieties of Taiwan were measured in separate plates with ‘Williams 82’ (one copy), ‘Peking’ (three copies), and PI 88788 (nine copies) and/or PI 209332 (ten copies) included in each plate. Relative fluorescence units (RFU) estimated by the BioRad CFX Maestro version 2.0 software were collected in R environment 4.0.2 to assess the variations among plates and soybean varieties. Technical measurements of each four biological replicate were averaged, and the four biological replicates were used to calculate the mean and variance representing each soybean variety. The 99% confidence interval was estimated using the R package “ellipse” version 0.4.2, and the RFU scatterplot was graphed in R to visualize the similarity among the soybean varieties.

2.3. Copy Number Analysis

The qPCR signal division of VIC (Glyma.18G-specific probe) and FAM (Glyma.11F-specific probe) of each variety was used in a linear regression analysis and linear model prediction. Repeated measurements on ‘Williams 82’, ‘Peking’, PI 88788, and PI 209332 were performed to ensure the variation among plates were negligible, and a total of 24 measurements on ‘Williams 82’ from six plates, 24 measurements on ‘Peking’, 12 measurements on PI 88788 from three plates, and 20 measurements on PI 209332 from five plates were included to build the linear model. For Gaussian linear regression, the Shapiro–Wilk test was used to evaluate the assumption of normality. Box–Cox transformation was performed to fulfill the normality requirements in modeling linear regression. Considering the rhg1 copy numbers present as discrete values rather than continuous values, the generalized linear models using Poisson regression and negative binomial regression were applied. The Akaike information criterion (AIC) and Bayesian information criterion (BIC) were computed between Gaussian model, Poisson model, and negative binomial model. The model with the less AIC and BIC values was regarded as the best model and used for predicting the rhg1 copy number in the 21 soybean varieties of Taiwan. The prediction values of each soybean variety of Taiwan were pairwise compared to the prediction values of ‘Williams 82’, ‘Peking’, PI 88788, and PI 209332 using the students’ t test, and the significance was claimed at Bonferroni-adjusted p value of 0.01.

2.4. Sequence Polymorphisms of the rhg1 Locus in the Soybean Varieties of Taiwan

A forward primer (5’-CTA GTT AGA GCA TGA ACT GC) and a reverse primer (5’-GTA ACA GGG CTA TCA C) were used to amplify the genomic region harboring two single nucleotide polymorphisms (SNPs) at the 10,978 and 10,995 position to distinguish the ‘Williams 82’ type rhg1-c, the ‘Peking’ type rhg1-a, or the PI 88788 type of rhg1-b locus [26]. The PCR was conducted using the high fidelity Phusion polymerase with HF buffer (New England Biolabs, Ipswich, MA, USA), and the PCR results were confirmed to be a single amplicon using 2% gel electrophoresis before direct submission to Sanger sequencing by the Tri-I Biotech Inc. (New Taipei City, Taiwan). The sequences of ‘Williams 82’, ‘Peking’, PI 88788, PI 209332, and the 21 soybean varieties of Taiwan were aligned using the Clustal Omega software.

3. Results

3.1. Relative Fluorescent Units (RFU) of rhg1 Copy Numbers in the Soybean Varieties of Taiwan

The modified qPCR conditions were used throughout the study to fit the instrument difference. While previous studies have confirmed the rhg1 copy number of PI 88788 and PI 209332 were nine and ten, respectively [15], this study resulted in a 99% confidence interval overlapping for these two varieties. Similar phenomenon was reported in the
original publication and the fluorescence difference between PI 88788 and PI 209332 was not significantly different from each other [27]. Although these results indicated the resolution limits of TaqMan qPCR on soybean varieties with high copies, the method was applicable to distinguish the F type (PI 88788, nine copies of \(rhg1-b\)), P type (“Peking,” three copies of \(rhg1-a\)), and the W type (“Williams 82,” one copy of \(rhg1-c\)), for which the 99% confidence intervals separated clearly from each group (Figure 1). The modified TaqMan method was applied to the 21 soybean varieties of Taiwan, and the RFUs of the 21 soybean varieties scattered closely to the W type locus, and none of the 99% confidence interval overlapped with “Peking” or PI 88788 or PI 209332. Accordingly, based on the relative fluorescence of VIC and FAM, the results indicated the possible grouping of the 21 soybean varieties of Taiwan to the susceptible variety “Williams 82.”

**Figure 1.** Detection of relative fluorescence unit (RFU) among soybeans with different copy numbers of \(rhg1\) locus. ‘Williams 82’ served as the susceptible soybean variety, which contains only one copy number of \(rhg1\) locus. ‘Peking’, PI 88788, and PI 209332 served as the resistant soybean varieties, which contain three, nine, and ten copy numbers of \(rhg1\) locus. All soybean varieties developed in Taiwan were measured to have RFUs undistinguishable from ‘Williams 82’. Each data point was averaged from three technical replicates in the qPCR measurement. Each variety was represented by four biological replicates, which included five plants in one DNA extraction; in other words, the four data points of each variety were represented by a total of 20 soybean plants. The ‘Williams 82’, ‘Peking’, and PI 209332 were measured in every plate as a control, and therefore, 16 data points were shown in the plot. The ellipse circles exhibit the 95% confidence interval for different soybean varieties.
3.2. Linear Modelling and Prediction of rhg1 Copy Numbers in the Soybean Varieties of Taiwan

In order to estimate the rhg1 copy number in the 21 soybean varieties of Taiwan, a Gaussian linear model using the ratio of VIC to FAM was built according to their copy number: one for 'Williams 82', three for 'Peking', nine for PI 88788, and ten for PI 209332. Due to the violation of normality (Shapiro–Wilk test, \( p = 2.535 \times 10^{-7} \)), the raw ratios of VIC to FAM were Box–Cox transformed and the transformed data were used to build the Gaussian linear model (Shapiro–Wilk test, \( p = 0.577 \)). As the transformation greatly resolved the problems in normality (Figure 2A), the Box–Cox-transformed linear model was accepted for rhg1 copy number prediction. In addition, considering the values of copy number are discrete integers rather than continuous values with all possible decimals, Poisson and negative binomial regression were modelled to better meet the statistical assumptions. The Gaussian, Poisson, and negative binomial models were selected based on the AIC and BIC statistics, and the results indicated that Box–Cox-transformed Gaussian linear model was the best regression based on the lower AIC and BIC value (Table 2).

Figure 2. Prediction of the rhg1 copy number in soybean varieties. (A) The quantile–quantile plot (Q-Q plot) showed the raw data of the relative fluorescent unit (RFU) ratio (VIC/FAM) violated the assumptions of normality. (B) The Q-Q plot showed that Box–Cox transformation evidently improves the normality violation, and the transformed data were subjected to the Gaussian linear modeling. (C) The rhg1 copy number prediction using the Gaussian linear model with Box–Cox transformed data successfully separated three resistant varieties including 'Peking', PI 88788, and PI 209332 from the susceptible variety ‘Williams 82’. None of the soybean varieties developed in Taiwan showed significant difference to ‘Williams 82’. The significance of student’s \( t \) test was declared based on the Bonferroni-adjusted \( p \) value at 0.01.
Table 2. Model selection using the Akaike information criterion (AIC) and Bayesian information criterion (BIC).

| Regression Model                      | AIC   | BIC   |
|---------------------------------------|-------|-------|
| Box–Cox transformed Gaussian linear model | 271.231 | 278.377 |
| Poisson model                         | 322.408 | 327.172 |
| Negative binomial model               | 322.784 | 329.930 |

Using the Box–Cox-transformed Gaussian linear model to predict the rhg1 copy number in the 21 soybean varieties of Taiwan, the results indicated all of these 21 varieties have rhg1 locus approximately to one copy, and none of these varieties showed significant difference to the ‘Williams 82’ (0.675 ± 0.247) in the student’s t test. On the other hand, the prediction on the ‘Peking’ (4.161 ± 0.372), PI 88788 (9.341 ± 1.581), and PI 209332 (8.792 ± 1.287) was all significantly different from the ‘Williams 82’ as well as the 21 soybean varieties of Taiwan. In summary, the linear prediction of rhg1 copy number in the 21 soybean varieties of Taiwan to be one single copy, which displayed no significant difference from the susceptible variety ‘Williams 82’ (Figure 2B).

3.3. Sequence Polymorphisms of the rhg1 Locus in the Soybean Varieties of Taiwan

Although a single copy of rhg1 indicates the likelihood of SCN susceptibility, sequence polymorphism could still present in the single copy to differentiate the wild type rhg1-c (‘Williams 82’), rhg1-a (‘Peking’), and rhg1-b type (PI 88788). The possibility of a single rhg1-a or rhg1-b, rather than the wild type rhg1-c, presents in the soybean varieties of Taiwan should not be ruled out. In order to assess the sequence polymorphism, the genomic region flanking two single nucleotide polymorphisms (SNPs) of rhg1 locus, the SNP-p of ‘Peking’ representing the rhg1-a, and the SNP-f of PI 88788 representing the rhg1-b (Lee et al., 2015), were amplified and sequenced. Unfortunately, the single copy rhg1 locus in all 21 soybean varieties of Taiwan were found to be identical to the susceptible variety ‘Williams 82’ and therefore the genotypes were determined as rhg1-c (Figure S1).

4. Discussion

This study applied the Taqman qPCR method to quantify 21 soybean varieties of Taiwan, and the results revealed that these 21 varieties of Taiwan have only one copy of rhg1 locus. Although SCN resistance can also be mediated by low copy numbers of rhg1, the allelic types of rhg1 appears to be critical in incorporating with the Rhg4 resistance. For example, in the resistant soybean varieties with the rhg1-a locus such as ‘Peking’, PI 567516C, and PI 437564, the copy numbers were estimated to be 1.9 to 3.5 incorporating with 1 to 4.3 copies of Rhg4-a, Rhg4-b, or Rhg4-c [17]. On the other hand, for soybean varieties with the rhg1-b locus such as PI 88788 and PI 548316, it was suggested that a minimal of 5.6 copies of rhg1-b would be needed [17]. There has been no soybean variety containing a single copy of rhg1-c found to be SCN resistant; and therefore, the results of this study indicated that the 21 soybean varieties of Taiwan are likely susceptible to SCN.

Due to the absence of SCN in Taiwan, genotyping becomes the practical method to evaluate SCN resistance for the soybean varieties developed in Taiwan. While there were some publications claimed the presence of SCN in Taiwan [29–32], these literatures seemed to misunderstand an old literature [33] probably due to the language inconvenience. In the Hung’s publication (Supplementary File 1), a table on the page 95 and the corresponding notes on the page 96 summarized several nematodes found in different fields of Pingtung County in Taiwan, which were based on the morphological characteristics. These nematodes included Aphelenchus sp., Criconemoides rusticum, Helicotylenchus sp., Meloidogyne sp. Pratylenchus sp., Rotylenchus sp., Trichodorus sp., and Xiphinema sp. The key words of SCN and Heterodera glycines showed up in the page 27 for discussion, where it stated “Heterodera glycines Ichinohe is absent in Pingtung, and the only sample collected in the Hsinchu Agricultural Research and Extension Station by Mr./Ms. Chang-Hsuan Hung of the Taiwan Agricultural Research Institute was a specimen” (translated from Man-
Accordingly, there appears to have been no living record of SCN ever being confirmed in Taiwan. More recently, an article entitled “The occurrence and management of plant parasitic nematodes in recent years (translated from Mandarin)” stated the absence of SCN in soybean fields even after several intents of field scouting [34]. Consistently, a thorough nematode survey conducted during 2015–2019 in Taiwan did not discover SCN [35], and field scouting for soybean diseases in Hualien County, Taoyuan County, Kaohsiung City, and Pingtung County in 2019 and 2020 found no SCN.

Because nematode are microscopic worms, the species identification traditionally is based on morphology and body measurements only. For the identification of cyst nematode, the key characters are the vulval cone of the cyst, the length of stylet, tail and hyaline tail terminus of the second stage juvenile [36]. SCN belongs to the schachtii group of *Heterodera,* and is morphologically very similar to *H. schachtii,* thus accurate identification requires considerable experience. Therefore, some regions are left with unconfirmed status (CABI, May 24 2021; https://www.cabi.org/cpc) for SCN distribution. For example, SCN was reported in Egypt [37] but there is no further study to validate this early documentation. The record of Egypt in the Invasive Species Compendium of CABI is currently “Absent, Unconfirmed presence record(s),” which is the same situation for Taiwan. The absence of SCN in Taiwan together with the finding that there is no *rhg1* resistance in the soybean varieties of Taiwan highlights the risk of SCN invasion and destruction that should not be negligible.

5. Conclusions

In the past five years, importation of soybean from SCN-presenting countries including the US, Brazil, and Canada dominated the market needs in Taiwan (Supplementary Table S1). With the continuing demand, nematode quarantine becomes an important task to minimize the chance of SCN invasion to Taiwan. Besides maintaining SCN on the quarantine pest list, further active and systemic approaches from pre-boarder to boarder, such as requiring appropriate sanitation plan on imported soybean seeds, developing sensitive and specific molecular detection assays for border inspection, as well as establishing effective quarantine measures for emergent nematode mitigation, will reduce the risk of SCN invasion and population establishment in Taiwan. As this study confirmed the absence of *rhg1* resistance locus in the 21 soybean varieties of Taiwan, future breeding programs should focus on the introduction of high copy numbers of *rhg1-b,* *rhg1-a* with *Rhg4.* With the successful integration management in quarantine and resistance breeding, production of edamame/soybean in Taiwan could be sustainable and free from SCN devastation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11071346/s1, Table S1. Soybean seeds importation from countries with SCN infestation. Figure S1. Nucleotide polymorphism of the *rhg1* locus among 21 soybean varieties of Taiwan. Supplementary File 1. The Publication of Hung, Y., 1958. The red block indicates discussion section related to soybean cyst nematode.

Author Contributions: H.-X.C. conceived the project and established the analysis system. C.-C.H. executed DNA extraction and qPCR measurement. C.-C.H. and H.-X.C. performed the data analyses. K.-L.C. and C.-H.L. helped with the field scouting, soybean materials, and pedigree information. J.-i.Y. and H.-X.C. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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