ddRAD Sequencing: A Novel Arsenal Added to the Biosecurity Toolbox to Trace the Origin of Brown Marmorated Stink Bug, *Halyomorpha Halys* (Hemiptera: Pentatomidae)

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Abstract

Background

Brown marmorated stink bug (BMSB), *Halyomorpha halys* (Hemiptera: Pentatomidae) is native to East Asia but has invaded many countries in the world. It is a polyphagous insect pest and causing significant economic losses to agriculture worldwide. Knowledge on the genetic diversity among BMSB populations is scarce but is essential to understand the patterns of colonization and invasion history of local populations. Efforts have been made to assess the genetic diversity of BMSB using partial mitochondrial DNA sequences but genetic divergence on mitochondria is not high enough to precisely identify and distinguish various BMSB populations. Therefore, in this study, we applied a ddRAD (double digest restriction-site associated DNA) sequencing approach to ascertain the genetic diversity of BMSB populations collected from 12 countries (2 native and 10 invaded) across four continents with the ultimate aim to trace the origin of BMSBs intercepted during border inspections and post-border surveillance.

Result

A total of 1775 high confidence single nucleotide polymorphisms (SNPs) were identified from ddRAD sequencing data collected from 389 BMSB individuals. Principal component analysis (PCA) of the identified SNPs indicated the existence of two main distinct genetic clusters representing individuals sampled from regions where BMSB is native to, China and Japan, respectively, and one broad cluster comprised individuals sampled from countries which have been invaded by BMSB. The population genetic structure analysis further discriminated the genetic diversity among the BMSB populations at a higher resolution and distinguished them into five potential genetic clusters.

Conclusion

The study revealed hidden genetic diversity among the studied BMSB populations across the continents. The BMSB populations from Japan were genetically distant from the other studied populations. Similarly, the BMSB populations from China were also separated from the Japanese and other populations. Further genetic structure analysis revealed the presence of at least three genetic clusters of BMSB in the invaded countries, possibly originating from multiple invasions. Furthermore, this study has produced novel set of SNP markers to enhance knowledge of genetic diversity among BMSB populations and demonstrate a great potential to trace the origin of BMSB individuals for future invasion events.

Background

The brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål, 1855) (Hemiptera: Pentatomidae) is a polyphagous pest with a wide host range [1]. It can cause extensive damage to agricultural crops worldwide [2, 3], and in 2010 alone was responsible for a loss of more than 37 million USD in agricultural products in North America [4]. The native range of BMSB is China (including Taiwan), Japan, and the Korean peninsula [5-7]. To date, BMSB has been reported from more than 30 countries [8], including almost all states in the USA [2, 4], multiple countries in Europe [9-16] and Chile [17]. Climate modelling studies indicates its potential range to develop could even expand further that includes South and Central America, Southern Africa, Southern Australia, and the North Island of New Zealand [9, 18].

BMSB was first intercepted at the New Zealand border in 2005 [19], and since then the frequency of interceptions have been increasing, due to the rise of international travelling and trade [20]. There have been 1620 recorded interceptions of BMSB since 2005 at the New Zealand border [19]. In the past decades, BMSB has been invaded and established in a range of countries irrespective of the environmental conditions [2, 4, 10-17, 21]. Adaptive evolutionary changes and/or ecological adaptation in a new region has made this pest a successful global invader and its recent invasion history can shed light on that. However, an in-depth genetic information of BMSB at the population level is scarce. Such information on the genetic diversity of BMSB can enhance our understanding on their population structure and global invasion history. This could also assist in constructing a global genetic population structure of BMSB and develop a potential strategy to trace the country of origin for BMSB individuals intercepted at the border or in post-border scenarios in biosecurity settings.

Up to date, nearly all published studies for tracing the origin of BMSB utilised PCR based molecular methods and focused on small regions on mitochondrial DNA (mtDNA), such as the COI (Cytochrome c oxidase I) and/or COII (Cytochrome c oxidase II) genes [16, 21-24]. mtDNA is highly variable between species and can potentially provide sufficient resolution to identify genetic differences between species [25]. Since mtDNA is inherited maternally and lacks recombination, the resolution of mitochondria-derived genetic divergence is not sufficient to differentiate between individuals in a population [26]. Therefore, there is the need to study the genome-wide, high-resolution markers among BMSB populations from their native and invaded regions. The study will be able to discern genetically distinct populations thus allowing us to trace the geographical origin of BMSBs within an interception scenario. This calls for an innovative method to explore the genetic diversity within BMSB populations on a whole genome scale.

The detection of genetic markers is crucial for genetic diversity study. Recently, a high-throughput sequencing-based method (HTS) has replaced traditional gel-based experiment to discover genetic marker [27]. RADseq (Restriction-site associated DNA sequencing), is often applied for genome-wide SNP (single nucleotide polymorphism) identification in large genomes because of its relatively low cost and high-throughput [28]. The RADseq technique utilises one (or more) restriction enzyme to digest the whole genome into short genomic fragments that are then subjected to high-throughput DNA sequencing [28]. Restriction site-associated DNA markers provide a well-established basis for population genetics, as they are sensitive to both SNPs and insertion or deletion events (indels) in genomes [29]. So far, RADseq has been widely used in population genetic studies for many taxa including plants [30], and animals [28]. Double digest restriction-site associated DNA (ddRAD) sequencing is using two restriction enzymes to allow greater control of the genomic regions sampled for sequencing and allowing more reproducible recovery of sequenced regions [31]. Therefore, in this study, we applied ddRADseq to explore the genetic diversity among BMSB specimens collected from 41 populations across 12 countries.

Results

EcoR I-Msp I restriction enzyme pair was suitable for ddRAD sequencing

To select the most suitable restriction enzyme (RE) pairs for digesting BMSB genomes, *in silico* test using 15 combinations of RE against the BMSB scaffold were conducted. The prediction revealed that more than 100K fragments produced from most of the RE pairs selected except the pairs, MseI-MluCI, MseI-MluCI and EcoRI-PstI (Table 1). Since the genome used for the test was a scaffold, thus the simulation results might not reflect the real situation. A pilot ddRADseq *in vitro* experiment was conducted with genomic DNA samples derived from two BMSB individuals. Of the 15 pairs of REs used for the *in-silico* test, nine different pairs of RE were selected for ddRADseq. After the Hiseq run, approximately, 2 Gb of raw RADseq sequencing data were generated for each individual. EcoR I-Msp I restriction enzyme pair recovered the highest number of genetic variances (i.e. SNPs) after SNP high standard quality control (QC) filtering, thus selected as the most suitable pairs of restriction enzymes for digesting the BMSB genome via ddRAD sequencing (Table 1).

ddRAD sequencing statistics and SNPs estimation

In total, 399 ddRAD sequencing datasets were obtained from the BMSB individuals, which yielded 3.6 billion of raw paired end reads (2x150 bp) (min: 4 million, max: 40 million and median: 7.6 million). On average, 9 million reads were generated for each individual. Using quality-trimming of the sequence data, 387,629 SNPs were estimated from 399 BMSB individuals. A high standard QC criterion was applied for filtering the SNPs, and only those loci that were shared by all the individuals were retained. As a result, the sequences from 389 individuals passed QC with 1775 high confidence biallelic SNPs that were used for the subsequent analysis of genomic diversity and population structure.

Genetic clusters were observed among the BMSB populations

At least three genetic clusters comprising China, Japan, and the invaded countries (Austria, Chile, Georgia, Hungary, Italy, Romania, Serbia, Slovenia, Turkey, and the USA) were revealed by Principal component analysis (PCA) (Figure 1). All BMSB individuals from Japan formed an isolated cluster, whereas BMSBs collected from the invaded countries were genetically closer to those of China. Interestingly, one BMSB individual from Chile was clustered together with the Chinese populations whereas the rest of the individuals were mixed into the individuals from Europe/USA populations (Figure 1).

Individuals from the same geographical region were genetically linked

To further emphasise the outcome of genetic clustering pattern via principal component analysis, minimum spanning networks (MSN) were constructed using SNPs profile of each individual, and their genetic variability were visualised among the population lineages (Figure 2). The MSN showed that all the individuals from China are genetically linked together in the network, which also applies for the individuals from Japan (Figure 2). There was a genetic divergence among the BMSB individuals from native regions of China and Japan, while those of invaded countries were more closely related in the network. Similar to the PCA results, one individual from Chile was found in the same clade of the Chinese samples, suggesting that this BMSB specimen might have originated from a recent invasion from China. The rest of the Chilean sample were distantly related from those in China and Japan but were more closely related to the samples from the European/USA groups, indicating that those possibly originated from secondary invasions from European/USA regions. The MSN also showed that one individuals from Italy and three from Slovenia were genetically linked to the Chinese populations, whereas the rest from these two countries were more closely related to those from European and the USA, suggesting multiple invasion might have been occurred (Figure 2).

Genetic distance between native populations of China and Japan was relatively higher

Population genetic divergence in the form of pairwise F_{ST} revealed significant ($p < 0.05$) genetic difference (except for that between China and Serbia) among 12 geographical groups or countries, with F_{ST} value ranging from 0.00055 between BMSB populations from Hungary and Serbia, to 0.20842 between BMSB populations from Japan and Romania (Table 2). We also observed that the genetic distance between native

populations of China and Japan was moderately higher ($F_{ST}=0.08$) than that between the populations of China and many other BMSB-invaded countries, such as Slovenia ($F_{ST}=0.03792$). Similarly, the genetic distance between the invaded populations in the USA and Chile was relatively low ($F_{ST}=0.0393$) compared to genetic distance between BMSB populations in Chile and the native regions, China ($F_{ST}=0.09844$) and Japan ($F_{ST}=0.17654$). Moreover, the F_{ST} between the BMSB populations from the neighbouring countries was very small, for example, Turkey and Georgia ($F_{ST}=0.01651$), Austria and Slovenia ($F_{ST}=0.02033$), Hungary and Serbia ($F_{ST}=0.00055$) (Table 2). A Neighbour-Joining (NJ) tree constructed using the F_{ST} pairwise values among the individuals from the 12 countries revealed the similar relationships among the BMSB populations from the 12 countries (Figure 4). The tree depicted the overall relationships of the populations and showed that Chinese and Japanese populations were clustered together while the others were formed into a clade (Figure 4).

Five genetic clusters exist in the BMSB populations

Furthermore, insights into the BMSB genetic diversity were achieved by population genetic structure analysis using fastSTRUCTURE. This analysis expanded the results of PCA (Figure 1) and provided more in-depth clustering for the BMSB populations from the invaded countries. It predicts the presence of at least three genetic clusters within the BMSB-invaded countries (Figure 3). The first is of those populations from the USA, Italy, Chile, Turkey, Georgia, and Hungary, the second is formed by Romania, and the third cluster is formed by Slovenia, Austria, and Serbia. The BMSB populations from China and Japan were clearly separated from the invasive populations (Figure 3).

Discussion

To the best of our knowledge, this is the most comprehensive population genomic study so far to unravel the genetic diversity and population structure of BMSB. The study utilised ddRAD sequencing to enhance the knowledge of global BMSB diversity and invasion history. We identified a suitable restriction enzyme pair for genomic digestion of BMSB genome for ddRAD sequencing study, which will be useful in future application. The ddRAD data were analysed using a combination of approaches, including principal component analysis (PCA), phylogenetic analysis and population structure analysis to elucidate the population structure and genetic diversity among the BMSB populations. The present study unambiguously proved that the BMSB populations in the two native regions of China and Japan were genetically distinct. Many BMSB populations from the invaded countries were genetically closer to those of China. Conversely, the Japanese BMSB populations were isolated and showed genetically less similarity to those from the invaded countries. Overall, this study has provided a remarkable resolution in unravelling the population structure and estimating the genetic relatedness among the BMSB populations from a set of native and invaded regions of the world.

Genetics isolation between the BMSB populations from China and Japan

The PCA and MSN analyses showed that the Japanese BMSB populations were isolated from the other populations studied here. The fastSTRUCTURE analysis showed that population structures of the BMSB samples from China and Japan were derived predominantly from different genetic components (Figure 3). These results suggested that there are genetic isolations for the BMSB populations between the two countries. Xu *et al.* [22] also observed genetic divergence between the BMSB populations from China and Japan via haplotype analysis of COII and 12S/CR gene regions. One possible explanation for such phenomenon could be the geographical distance between the isolated populations. The width of Tsushima Strait is from 41.6 km to 222 km which is the distance between mainland and Japan [34]. However, the average non-stop flight distance of BMSB is less than 1 km [35]. Therefore, active spread of BMSB individuals from China to Japan is unlikely.

Estimation of the BMSB invasion pathway

BMSB populations from the invaded countries are more closely related to China than that of Japan. For example, the USA populations with F_{ST} of 0.084 to the Chinese populations and 0.16 to the Japanese populations, indicating that the USA populations originated more likely from China, but Japan. The results are consistent with the findings of a previous study [36]. Moreover, there were large genetic differences ($0.15 < F_{ST} < 0.25$) between the BMSB populations in Japan and the invaded countries of Chile, Georgia, Italy, Romania, Turkey, and the USA (Table 2). Therefore, we can conclude that the BMSB populations in the six invaded countries might not have been originated from Japanese populations.

Moreover, PCA plot (Figure 1, Additional file 3) indicated that the established populations in Chile, Hungary, Georgia, Italy, Turkey and the USA belonged to one genetic cluster. The fastSTRUCTURE analysis supported this point that those populations had similar genetic components (Figure 3). The neutrality test (Fu's F_s) in a previous study (Yan *et al.* unpub.) for these populations was negative ($p < 0.05$), indicating that these established populations have been under population expansion stage [37].

One individual from the Italy cohort was genetically closely related to the Chinese population (Figure 2), suggesting the possible recent arrival of that specimen from China. Besides, the rest of the BMSB samples from Italy showed their close relatedness to the USA and other European populations, suggesting that multiple BMSB invasions have occurred in Italy in the past [16, 38]. Such phenomenon was also observed in Chile

[17]. Most of the BMSB specimens from Chile, except one individual, were genetically close to each other (Figure 2), implying that there were at least two separated invasions of BMSB in Chile.

Predication of the BMSB genetic cluster

The population structure analysis of BMSB indicated that there were at least five genetic clusters identified among the BMSB populations studied here. Besides the Chinese and Japanese BMSB populations, there were three genetic clusters in Europe, the USA and Chile. The analysis showed that the BMSB populations from the USA, Georgia, Turkey, Italy, Hungary, and Chile were more likely belong to one genetic cluster due to their similar genetic structure. Since first detected in the USA in 1996 [39], BMSB has invaded many countries. In Europe, BMSB was first recorded in Switzerland in 2004 [14, 21, 40], then spread to many countries such as Germany, France, Italy and Hungary [41, 42]. Therefore, based on the timeline of reports, BMSB invasion might have started in the USA (1996) [2], then Europe (2004) and Chile (2017) [17], though a more transparent history of invasion remains unknown and needs further investigation.

Another interesting phenomenon is that the genetic distances between BMSB populations originating from Slovenia, Austria, and Serbia, where the species had been reported more recently, and the Chinese populations were smaller than those between the Chinese and the other European populations. This suggests that BMSB found in these three European countries could have recently been invaded directly from China rather than from one of their neighbouring countries in Europe. In saying that, the population genetic structure analysis also showed that the BMSB populations from Slovenia, Austria, and Serbia contained the components of the Europe-established BMSB populations (Figure 3). This analysis indicated that there were inbreeding between the between the BMSB individual from the adjoined established European BMSB populations and the Chinese populations. The principal component analysis also supported this as these populations were located between Chinese populations and other established BMSB populations from Europe. This result indicated that these BMSB populations were still under early stage of invasion, which supports the findings of haplotype analysis using COI and COII of BMSB samples from the same populations (Yan *et al.* unpub.).

BMSB was first reported in Romania in 2015 [10] and have spread around the country [43]. The F_{ST} values between the BMSB samples from Romania and the two native countries, China, and Japan, were 0.13334 and 0.20842, respectively, which were larger than the F_{ST} values from other invaded populations. The population structure analysis showed that Romanian BMSB samples had a unique genetic structure, which indicates that this group could have been invaded from a place of origin that was not covered in this study. Therefore, this study further emphasised the importance of sampling. Overall, this study covered a wide range of areas including wide sampling from the BMSB native regions and the invaded countries, although we still miss samples from some countries, such as Korea, Greece, Canada and Switzerland. Therefore, further study of ddRADseq of BMSB samples from those countries will provide more insights into BMSB genetic diversity of the BMSB populations.

Novel genetic analysis for the BMSB populations from the invade countries

Nevertheless, this study also provided novel genetic information on the BMSB populations for some of the European countries, such as Austria, Serbia, Slovenia, and Georgia. Since BMSB was detected in Austria [34] and Serbia [35] in 2015 and Slovenia [36] in 2017, Turkey [37] in 2019 and Georgia [38] in 2016, to the best of our knowledge, no genetic studies have been reported on those populations, and this study shows the first effort to characterise the BMSB populations for these countries. The results showed that the BMSB populations from Georgia and Turkey were more likely the next generation of the BMSB from the USA whereas those populations from Austria, Serbia and Slovenia were the hybrids of Chinese and USA populations (Figure 3). The results obtained here will be useful for future monitoring of the pest and assessing the potential pathways of invasion in those countries.

Finally, in compassion with the mitochondrial based (i.e. COI and COII) haplotypes analyses, here we show that ddRADseq can provide high resolution in identifying BMSB genetic populations. As an alternative, analysis of genome-wide SNPs via whole genome sequencing can be applied for deeper understanding the BMSB genomes and genetic diversity, however, it is still more expensive due to the large size of the BMSB genome (~1 Gbp) (Hhal_2.0). Therefore, cost effective approach such as the RAD-based approach with restriction enzymes (ddRAD) used in this study can show a higher resolution in revealing genomic diversity and population structure among BMSB populations. Taken together, we recommend applying genome-wide SNP markers-based study using ddRAD to explore the genomic diversity in insects and other eukaryotic organisms in future applications. Most importantly, our study will help to trace the geographic origin of the BMSB samples, irrespective of their life stages, and sex, that are intercepted at the New Zealand border. The advancement and findings resulted from our study will not only help in claiming the BMSB freedom status to New Zealand but also will greatly assist in the decision making during an incursion and response situation.

Conclusions

This study demonstrates that the restriction enzymes pair of EcoRI and MspI is suitable for generating restriction-associated DNA fragments for identifying SNPs within the BMSB genome through ddRAD sequencing. Via population analyses using ddRADseq data we detected genetic differentiations among different BMSB populations studied in both the native and invaded countries. We believe that this study could also assist in studying the invasion history of BMSB populations and tracing the potential geographical origin of unknown BMSB intercepted at the border or in post-border scenarios in biosecurity settings using the population structure model. Thus, ddRAD sequencing going to be an invaluable arsenal added in to the biosecurity toolbox for tracing the geographic origin of insects at the border/post-border in the near future.

Methods

BMSB specimen collection and DNA extraction

BMSB samples were collected from 41 regions in 12 countries, namely Austria, Chile, China, Georgia, Hungary, Italy, Japan, Romania, Serbia, Slovenia, Turkey, and the USA (Table 3, Additional file: 1). The field-collected samples were preserved in plastic vials containing 95% ethanol and imported into New Zealand in accordance to the Import Health Standard, Section 22 of the Biosecurity Act 1993. All the specimens were identified by the collectors and confirmed by the Entomologists at PHEL. No specific permits were required for all the sample collected from all the countries covered in this study. Ethics approval was not required as insects are not classified as animals for the purposes of the Animal Welfare Act, 1999, New Zealand Legislation.

Total genomic DNA was extracted from each individual using QIAGEN DNeasy® Blood & Tissue Kit with QIAGEN RNase A treatment (Qiagen, Valencia, CA, USA). Briefly, head and thorax or abdomen was ground using sterile plastic pestle in 1.5 mL tube with ATL buffer and Proteinase K added, and then incubated at 56 °C overnight. The DNA extraction followed the manufacturer's instructions and the DNA was eluted in 150 µl AE buffer. DNA quality was assessed using NanoDrop™ spectrophotometer (ThermoFisher Scientific, USA) and quantified using QuantiFluor™ system (ThermoFisher Scientific, USA). It was followed by an assessment for DNA shearing on a 1% agarose gel against 1Kb Plus DNA ladder (Invitrogen™, CA, USA) in TAE buffer stained with SYBR safe (Life Technologies, CA, USA) and visualised using a Gel Doc Software system (BioRad, Hercules, CA, USA).

Selection of restriction enzymes *in silico* for RAD sequencing

The selection of restriction enzyme (RE) is the crucial step for experimental ddRAD-seq analysis and discovery of the SNP across the genome for genetic diversity study, thus initial tests on the RE used for the library was conducted. To select the best RE, a simulation based on the BMSB reference genome (submitted as Hhal_2.0 by The i5k Initiative on 15/12/2017) and 15 combinations of 9 enzymes (AvaII, EcoRI, MseI, MspI, NlaIII, PstI, SbfI, SphI, MluCI). A Python script was developed to calculate the number of segments after using different combination of restriction enzymes to cut reference genome.

Selection of restriction enzymes *in vitro*: RAD sequencing and bioinformatics analysis

Nine pairs of enzymes which had higher numbers of segments in *in silico* analysis or had been used in other published studies [31] were selected. To further confirm the suitability of the RE pairs, two DNA samples were selected to be used for ddRADseq as parallels to eliminate the effects from individual difference. Eighteen RADSeq libraries (9 pairs of enzymes for 2 samples) were prepared using Illumina® TruSeq DNA Nano following the manufacturer's instructions and ToBo lab ezRAD Protocol-v3.2 [49]. Before sequencing, all RADSeq libraries were assessed using a Fragment Analyzer for quality control. The sequencing was conducted by Annoroad Gene Technology Co., Ltd (Beijing, China) on a HiSeqX ten sequencing platform (paired end, 2x150 bp). The 3'end adaptors of raw reads were trimmed using AdaptorRemoval v2 [50] and reads with phread quality score of less than 20 and length of less than 50 bp were discarded using an in-house script. Then, quality-trimmed sequence datasets, were mapped to the BMSB reference genome, Hhal_2.0, using BWA version 0.7.17 [51] default setting and files with the mapping information (i.e. in SAM format) were converted to BAM format, sorted and indexed using SAMtools version 1.9 [52] before identifying the SNPs. SNPs were called using GATK v4.1 [53]. A hard filtering was conducted to discard the false positive SNPs as recommended by GATK developers (QD (QualByDepth) < 2.0, FS (FisherStrand) > 200.0, SOR (StrandOddsRatio) > 10.0, MQRankSum (MappingQualityRankSumTest) < -12.5, ReadPosRankSum (ReadPosRankSumTest) < -8.0). Population level quality control was done using Plink 1.9 (parameters used: call rate 0.8) [54] for subsequent analysis. All bioinformatics analysis was performed on the high-performance computing platform of the New Zealand eScience Infrastructure (NeSI), Auckland, New Zealand.

Library preparation and ddRAD sequencing

After selecting the most suitable pairs of restriction enzymes (EcoRI and MspI) for digestion of BMSB genome via RADseq approach, 399 BMSB individuals were further subjected to double-digest restriction-associated DNA (ddRAD) sequencing. ddRAD libraries were prepared using 500 ng of genomic DNA from each sample following the protocol described by Peterson *et al* [31] with some modifications. Briefly, genomic DNA was digested at 37 °C for 5 h using 10 Units of the two selected restriction enzymes, namely EcoRI and MspI (NEB, MA, USA), and deactivated at 65

°C for 20 mins, then followed by the ligation of Illumina adapter sequences and unique 8 bp barcodes, which varied by at least three bases. Sets of 24 differentially barcoded individuals were pooled and run on a 1% agarose gel, where 220~450 bp fragments were manually excised and purified using a Zymoclean™ Gel DNA recovery kit (Zymo Research, CA, USA). Each pool was amplified by PCR reactions, which were carried out at a total volume of 25 µl, each containing 5 µl 5×Reaction buffer, 5 µl 5×High GC enhancer, 0.25 µl Q5 polymerase, 5nM of library DNA and a unique indexing primer for each pool that corresponds to the standard Illumina multiplexed sequencing protocol. Temperature cycling for PCR comprised one initial denaturation at 98 °C for 30 s, followed by 14 cycles of denaturation at 98 °C for 15 s, annealing at 65 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 mins. The PCRs were carried out in a Veriti 96-well thermal cycler (Life Technologies). DNA libraries were quantified using the Agilent high-sensitivity DNA kit in a 2100 Bioanalyser (Agilent Technologies, CA, USA). Library pools were combined in equimolar concentration to form a single genomic library and sequenced in one lane of a HiSeqX ten Illumina sequencer (paired-end, 2×150 bp) by Personalbio co. (Shanghai, China)

Sequencing data pre-processing and Quality control of ddRAD data

To pre-process the raw sequencing data generated via ddRAD, a similar pipeline and data quality filtering approach was applied as described in the section of processing of RADSeq data. In order to call SNPs from ddRADseq data, the raw fastq files were mapped to reference genome, Hhal_2.0, to produce a mapped read file (sam file) using Burrows-Wheeler Aligner v 0.7 [51]. The result was converted into binary format (BAM files) and sorted to increase calculation speed for further analysis using Samtools v1.9 [54]. SNPs were called from BAM files using GATK4 [53]. The quality control parameter used here was the same as that used for the selection of restriction enzymes above. In addition, the obtained SNPs were further filtered using SNP call rate of 1 and MAF (minor allele frequency) of 0.03 using Plink 1.9 [54]. As a result, 10 samples were discarded due to lower SNP call rate.

Population Genomic Analysis

Using the SNPs identified from the 389 BMSB individuals, we conducted principal component analysis (PCA), neighbour-joining tree (NJ tree) and population structure analysis using fastSTRUCTURE to elucidate the population structure and genetic diversity among the BMSB populations. PCA was conducted based on with 1775 SNPs obtained from 389 individuals using Plink 1.9 [54] (parameters used: `--allow-extra-chr --pca`) and plotted using Tableau 2019 [55]. To further explore the genetic relatedness among BMSB individuals, a Minimum Spanning Network based on the SNP results of each individual was also reconstructed using R package poppr [56].

To test for the presence of population structure, pairwise F_{ST} values were generated from the ddRAD data by combining the individual data from the same country as one geographic group. This analysis was implemented in Arlequin 3.5 [57] by converting the SNP profile (in Plink format) to Arlequin format using PGDSpider 2.1.1.5 [58]. The obtained population average pairwise F_{ST} values were further used to construct a Neighbour-Joining tree in MEGA X 10.1 [32] to reveal the population level's genetic distance.

To provide additional insight into the genetic variation and population differences, population genetic structure analysis was conducted. The population genetic structure was inferred using the SNPs profile of individual samples. In this process, fastSTRUCTURE 1.0 [59] was used to conduct model-based clustering of all individuals. The best K value (i.e. the number of populations or clusters that the samples are best divided into) was determined as 5 using a python script, chooseK.py within the fastSTRUCTURE software.

Abbreviations

BMSB: brown marmorated stink bug

mtDNA: mitochondrial DNA

COI: Cytochrome c oxidase I

COII: Cytochrome c oxidase II

RADseq: Restriction-site associated DNA sequencing

ddRAD: double-digest Restriction-Associated DNA

SNPs: Single Nucleotide Polymorphisms

PCA: principal component analysis

MSN: Minimum spanning networks

NJ tree: Neighbour-Joining tree

Declarations

Ethics approval and consent to participate

No specific permits were required for all the sample collected for this study. No samples were collected in national parks and no endangered or threatened insects were included in this study, thus collection permits were not required for all the collections. All specimens imported into New Zealand were in accordance to the Import Health Standard, Section 22 of the Biosecurity Act 1993. Ethics approval was not required as insects are not classified as animals for the purposes of the Animal Welfare Act, 1999, New Zealand Legislation.

Consent for publication

Not applicable

Availability of data and materials

The SNP data can be found using the link below

<https://1drv.ms/u/s!AvRFcQuxR5slgpcYfFCZ3C3pSAa1Xg?e=L7PmSe>

Competing interests

The authors declared that they have no competing interests.

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Author's contributions

DL, CP, DA and SG conceived the study. DL, JY, CP, AB, and RKB designed the study. GV, PF, JZ and RG collected and provided the BMSB specimens. QF and DG performed the morphological identification. JY conducted all the laboratory experiments and sequence analysis. CP provided the bioinformatics support. JY drafted the manuscript with input from DL and CP. All authors have read, edited and approved the final version of the manuscript.

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Tables

Table 1 Summary of the *in silico* and *in vitro* tests of RE pairs for ddRADseq.

RE pairs	<i>In silico</i> : numbers of Segment *	<i>In vitro</i> : numbers of SNPs **
MspI-NlaIII	556,040	969
EcoRI-NlaIII	508,739	311
PetI-NlaIII	481,590	Null
MseI-PstI	303,697	17
EcoRI-MseI	296,739	20,136
EcoRI-MluCI	267,087	Null
MspI-MseI	265,748	27,871
PstI-MluCI	259,942	Null
MspI-MluCI	222,963	16,369
MseI-NlaIII	182,338	3,909
NlaIII-MluCI	148,911	2,135
EcoRI-MspI	118,867	28,328
MseI-MluCI	86,829	Null
MspI-PstI	86,012	Null
EcoRI-PstI	31,417	Null

Note: * Prediction of the DNA segments of 300-500 bp against the BMSB genome scaffold

** Two replicates were used for each RE pairs. The number showed the shared SNPs between two replicates. Null indicates not tested.

Table 2. The group pairwise F_{ST} (Fixation index) between the BMSB populations from 12 countries

	Japan	China	Hungary	Serbia	the USA	Chile	Slovenia	Italy	Romania	Turkey	Austria
China	0.08473										
Hungary	0.14372	0.0639									
Serbia	0.1338	0.04756*	0.00055								
the USA	0.16267	0.08428	0.01295	0.01226							
Chile	0.17654	0.09844	0.03937	0.0405	0.0393						
Slovenia	0.10275	0.03792	0.02487	0.01052	0.04199	0.06715					
Italy	0.15174	0.07859	0.02627	0.02892	0.03944	0.04412	0.03764				
Romania	0.20842	0.13334	0.0609	0.06616	0.05928	0.09133	0.09115	0.07734			
Turkey	0.17471	0.09696	0.03322	0.02977	0.01641	0.05674	0.06398	0.05738	0.0937		
Austria	0.13983	0.06013	0.04398	0.03359	0.06301	0.0944	0.02033	0.0657	0.11817	0.09175	
Georgia	0.16825	0.0901	0.02413	0.02107	0.01465	0.05951	0.05157	0.04188	0.07368	0.01651	0.07778

Note: Asterisk (*) indicates not statistically significant difference ($p > 0.05$). F_{ST} value ranges from 0 to 1, where 0 means no genetic difference (i.e. similar) and 1 means high difference (isolated populations). Values close to zero indicate the populations are sharing their genetic structure and has minimal difference between them.

Table 3 Sample metadata of BMSB collection. N represents the number of individuals.

Country	Locality	N
Austria	Vienna City	16
Chile	Santiago City	19
China	Anhui Province	6
	Beijing City	26
	Guizhou Province	1
	Hainan Province	7
	Hebei Province	8
	Jilin Province	3
	Shaanxi Province	27
	Shanxi Province	9
Georgia	Eki City	30
	Samegrelo-Zemo Svaneti Region	3
Hungary	Budapest City	39
	Debrecen City	10
	Pécs City	6
	Szeged City	3
	Szombathely City	12
Italy	Codroipo (UD) Comune	4
	Mantua City	6
	Pozzuolo Del Friuli (UD) Comune	11
	Trentino-Alto Region	1
Japan	Akita Prefecture	3
	Chiba Prefecture	2
	Gifu City	2
	Ibaraki Prefecture	2
	Ishikawa Prefecture	2
	Iwate Prefecture	5
	Kagoshima City	8
	Kanagawa Prefecture	3
	Kyoto City	10
	Mie Prefecture	5
	Miyagi Prefecture	3
	Saga Prefecture	3
	Shizuoka City	7
	Yamanashi Prefecture	12
Romania	Bucharest City	22
Serbia	Senta Town	10
Slovenia	Ljubljana City	16
Turkey	Arhavi Town	12

the USA	Maryland State	16
	West Virginia State	9
Sum		399

Figures

Figure 1

Principal component analysis (PCA) plot using 1775 SNPs of 389 individuals. Each point represents an individual. The colour represents the country where the individuals were collected from. X axis represents the variance explained by PC2 (10.3%), and Y axis represents the variance explained by PC1 (28.7%). The picture was created using Tableau 2019 (<https://www.tableau.com/>) [55]

Figure 2

Minimum spanning networks (MSN) of BMSB individuals. The analysis was based on 775 SNPs derived from 389 individuals of 12 geographical groups comprising Austria, Chile, China, Georgia, Hungary, Italy, Japan, Romania, Serbia, Slovenia, Turkey, and the USA. Each node represents an individual specimen and the edge indicates the genetic distance (dissimilarity: fast pairwise distances) between the individuals. The colour in each circle represents the countries where the samples were collected from. The picture was created using R package poppr [56].

Figure 3

The fastSTRUCTURE bar plot showing the genetic clusters. The assignment probabilities ($K=5$; the number of populations or clusters that the samples are best divided into) of each genotyped individual of BMSB from 12 different geographical regions were shown. Each pixel column is one individual sample. Different colours in a bar represent proportion of the genomic component in a sample derived from different populations. Dotted line was used to divide BMSB populations from different countries.

Figure 4

The Neighbour-Joining (NJ) tree of 12 geographical groups. The phylogenetic tree was constructed using MEGA X [32] based on genetic distances of population pairwise F_{ST} values. The tree shows the evolutionary history of each BMSB population [33]. The optimal tree with the sum of branch length = 0.30043098 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The branch lengths are showed in each node. Scale bar shows the evolutionary time between two nodes and represents the substitutional per sequence site.

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