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Genetic Diversity and Population Structure of *Bursaphelenchus xylophilus* in Central China based on SNP Markers

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Abstract: Hubei, Hunan and Henan Provinces are located in Central China, a region with extensive transport networks and trade. *Bursaphelenchus xylophilus*, the causative agent of pine wilt disease, is spreads mainly through human activities. In order to further understand the genetic structure of PWN in Central China, we studied the genetic information of PWN populations in this region and compared the genetic relationship with strains from Guangdong and Jiangsu provinces. We found that the HB (Hubei) 15, HEN (Henan) 20, HN (Hunan) 07, HN08 and HN10 had significantly more SNPs and homozygotes than other strains from Central China, and their most frequent mutant genotypes also differed from other strains. The clustering results indicated that HB15, HEN 20, HN07, HN08 and HN10 were genetically distinct from other strains and closely related to Guangdong strains. We also observed significant genetic variation among strains in Henan province, suggesting that some of them might have different transmission sources than those from Hubei and Hunan provinces. The introgression analysis was conducted and identified three possible pathways: 1) Guangdong to Henan; 2) Guangdong to Hunan; 3) Jiangsu to Hubei. The results provide a basis for tracing the origin and spread of PWD in China.

Keywords: *Bursaphelenchus xylophilus*; SNP; genetic diversity; population differentiation

1. Introduction

Pine wood nematode (*Bursaphelenchus xylophilus*, PWN) is the causal agent of pine wilt disease (PWD), which poses a hazard to pine forests throughout Europe and Asia. PWN is regulated as a quarantine disease in most countries due to its ecological and economic impacts [1]. Previous studies have indicated that China is one of the most affected countries by PWD, with most provinces being suitable for PWN establishment and spread [2]. As an invasive alien species, PWN has caused significant loss to the pine forests in China. It was first detected in Nanjing, Jiangsu province in 1982, and since then it has expanded to 731 county-level administrative regions of 19 provinces (National Forestry and Grassland Administration No. 6 of 2022). This suggests that PWN has a wide distribution in China, and that human activities such as transportation of infested wood and infrastructure construction are the main pathways of its dissemination. However, the transmission routes of PWN in China are not well understood due to the difficulties and limitations of monitoring and surveillance. In recent years, many studies have focused on the early diagnosis of PWN, which provides a theoretical basis for the prevention and control of PWD [3-5].

Studies have shown that the critical time to control biological invasions is in the early stage [6, 7], and studying the dispersal path of invasive organisms is crucial to achieving control. Several studies have also proved that the inference of invasion pathways provides information about the biological invasion process, which enables us to understand the

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ecological characteristics of invasive populations and is helpful for control or eradication [8-11]. In order to clarify the transmission path and population differentiation of PWNs, relevant scholars have used different molecular marker technologies to analyze the population genetic structure of PWNs in some geographical areas [12-15].

As early as 2007, RAPD-PCR was used to analyze the genetic variation of Spanish PWN [16]. Subsequently, Valadas et al. used ISSR molecular markers to analyze the genetic differences among 43 PWN strains from five countries: China, Japan, Korea, the United States and Portugal [17]. With the development of molecular marker technology, SNP is considered the most promising molecular marker. It is widely used in many applications with population tracking, molecular genetics and disease diagnosis [18-21]. In recent years, studies on the population diversity of PWNs using SNP have also been reported [13, 22, 23]. Joana Figueiredo et al. used SNP labeling technology to analyze the differences of SNP in 7 PWNs from Portugal, China, the United States and Japan. It showed that the Portugal strains were closer to those from China, and the genetic distance between the American and the Japanese was relatively wide [15]. Several studies have analyzed the population diversity of PWN in different regions of China using SNP labeling technology. The genetic structure of Guangdong Province showed that it has high genetic diversity and multiple transmission sources [13]. Population results in eastern China showed that there was some correlation between each group and geographical origin [22].

Hubei, Hunan and Henan provinces are located in the middle of China, a region with frequent economic and trade activities. Therefore, supervising infected trees poses considerable challenges. Some scholars speculated that Guangdong Province was the initial colonization and diffusion center of PWNs in China, and Jiangsu Province was the new diffusion center [24]. These provinces are close to Hubei, Hunan and Henan, which were classified as epidemic areas in 2000, 2003 and 2009 respectively. To better understand the genetic structure of PWNs in central China, this study used whole genome resequencing and SNP molecular marker technology to analyze the genetic diversity of PWN populations in central China and explore their genetic relationship with PWN populations in Jiangsu and Guangdong provinces. This has great significance for establishing a PWD tracing system.

2. Materials and Methods

2.1. Isolation and purification of nematodes

Infected trees were collected from Henan Province, Hubei Province, Hunan Province, Guangdong Province and Jiangsu Province. Nematodes were extracted from chopped trees using the Baermann funnel method. Morphological identification of PWNs was carried out according to the characteristics of PWNs[25]. After DNA extraction, molecular identification based on the SCAR marker was performed to ensure detection accuracy[26].

After being identified as PWN, about 30 individuals were selected and cultured on the *Botrytis cinerea* at 28 ℃. When a sufficient sample size was obtained, nematodes were isolated using the Bermann funnel method and were cleared with 0.05 % streptomycin sulfate and sterile water for storage. All the strains were stored in the PWN Strain Resource Bank of the Forest Pathology Laboratory of Nanjing Forestry University.

2.2. Genome resequencing

The DNA of PWN was extracted by the CTAB method [26] and stored in the DNA Resource Bank of PWN, Laboratory of Forest Pathology, Nanjing Forestry University. Nano-drop, Qubit (Thermo Fisher) detected the DNA concentration and quality. The qualified DNA was sent to Wuhan Future Group Biological Company for high-throughput genome sequencing on the HiSeq 4000 platform. The genome resequencing method was 150 bp paired-end sequencing, and the average sequencing depth was greater than 40 ×.

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2.3. Identification and filtration of mutation sites

The quality of the raw data was first assessed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Filtered reads were aligned to the reference genome of PWN announced in 2022 [27] by BWA (http://bio-BWA.SourceForge.net/BWA.shtml). Samtools (http://samtools.sourceforge.net/samtools.shtml) and Picard were used to remove duplicates. Putative SNPs were called by Freebayes (https://github.com/ekg/freebayes) with minimum coverage (> 10), and VCFtools (https://github.com/vcftools) was used for SNP site statistical analysis.

2.4. Genetic differentiation analysis

The SNPs with low allele frequency, high linkage disequilibrium and missing rate were filtered by the SNPRealte package (https://www.bioconductor.org/packages/release/bioc/html/SNPRelate.html) of RStudio software (https://www.rstudio.com/). Principal component analysis (PCA) diagram was drawn using the same package mentioned above. PLINK (v1.9) (https://www.cog-genomics.org/plink/) was used to extract the filtered site information to generate new vcf file for phylogenetic tree analysis. VCF-kit (https://vcf-kit.readthedocs.io/en/latest/) and MEGA (v11.0.11) (https://www.megasoftware.net/) was used to construct phylo-tree using the neighbour-joining method.

Treemix software (https://bitbucket.org/nygcresearch/treemix), based on allele frequency as the basis for genetic distance calculation, was used to construct phylogenetic trees and label gene exchanges. Plink was used to calculate allele frequency for all SNP loci in advance, and the parameter "-noss-global" was used to construct a maximum likelihood tree.

3. Results

3.1. Sample collection

After purification and culture, 50 PWN samples from five provinces in China were obtained: Hubei (HB), Henan (HEN), Hunan (HN), Guangdong (GD) and Jiangsu (JS). Table 1 shows the sample information of 50 *B. xylophilus* strains.

Table 1 Sample information of 50 *B. xylophilus* strains

Strains No.	Origin	Host	Sampling time
GD02	Qingcheng District, Qingyuan City, Guangdong Province	*Pinus massoniana*	2015.01
GD04	Huiyang District, Huizhou City, Guangdong Province	*P. massoniana*	2015.01
GD08	Boluo County, Huizhou City, Guangdong Province	*P. massoniana*	2015.01
GD10	Huangpu District, Guangzhou City, Guangdong Province	*P. massoniana*	2015.01
GD14	Dongguan, Guangdong Province	*P. massoniana*	2015.01
GD17	Tianhe District, Guangzhou City, Guangdong Province	*P. massoniana*	2015.01
GD23	Meijiang District, Meizhou City, Guangdong Province	*P. massoniana*	2015.11
GD26	Fengshun County, Meizhou City, Guangdong Province	*P. massoniana*	2017.08
GD30	Haifeng County, Shanwei City, Guangdong Province	*P. massoniana*	2017.08
GD32	Dongyuan County, Heyuan City, Guangdong Province	*P. massoniana*	2017.08
HB01	Chibi, Xianning City, Hubei Province	*P. massoniana*	2015.04
HB02	Changyang County, Yichang City, Hubei Province	*P. massoniana*	2015.04
HB03	Enshi City, Enshi Prefecture, Hubei Province	*P. massoniana*	2015.04
HB05	Huangpi District, Wuhan City, Hubei Province	*P. massoniana*	2015.04
HB06	Huangpi District, Wuhan City, Hubei Province	*P. massoniana*	2015.04
HB07	Huangpi District, Wuhan City, Hubei Province	*P. massoniana*	2015.04

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HB08	Yiling District, Yichang City, Hubei Province	*P. massoniana*	2015.11
HB10	Yidu District, Yichang City, Hubei Province	*P. massoniana*	2015.11
HB12	Zengdu District, Suizhou City, Hubei Province	*P. massoniana*	2017.08
HB15	Luotian County, Huanggang City, Hubei Province	*P. massoniana*	2017.01
HEN02	Xin County, Xinyang City, Henan Province	*P. massoniana*	2015.08
HEN03	Xin County, Xinyang City, Henan Province	*P. massoniana*	2015.08
HEN04	Xin County, Xinyang City, Henan Province	*P. massoniana*	2015.08
HEN06	Xichuan County, Nanyang City, Henan Province	*P. massoniana*	2017.01
HEN09	Xichuan County, Nanyang City, Henan Province	*P. massoniana*	2017.11
HEN10	Xichuan County, Nanyang City, Henan Province	*P. massoniana*	2017.11
HEN14	Xixia County, Nanyang City, Henan Province	*Pinus tabuliformis*	2018.01
HEN15	Xixia County, Nanyang City, Henan Province	*P. massoniana*	2018.01
HEN19	Xin County, Xinyang City, Henan Province	*P. massoniana*	2018.01
HEN20	Xin County, Xinyang City, Henan Province	*P. massoniana*	2018.01
HN01	Cili County, Zhangjiajie City, Hunan Province	*P. massoniana*	2015.03
HN02	Linxiang City, Yueyang City, Hunan Province	*P. massoniana*	2015.03
HN03	Yunxi District, Yueyang City, Hunan Province	*P. massoniana*	2015.03
HN04	Hengnan County, Hengyang City, Hunan Province	*P. massoniana*	2015.03
HN06	Cili County, Zhangjiajie City, Hunan Province	*P. massoniana*	2015.08
HN07	Taoyuan County, Changde City, Hunan Province	*P. massoniana*	2016.08
HN08	Taoyuan County, Changde City, Hunan Province	*P. massoniana*	2016.08
HN09	Lingling District, Yongzhou City, Hunan Province	*P. massoniana*	2019.03
HN10	Shaoyang County, Shaoyang City, Hunan Province	*P. massoniana*	2019.03
HN13	Lingling District, Yongzhou City, Hunan Province	*P. massoniana*	2019.03
JS01	Liuhe District, Nanjing City, Jiangsu Province	*P. massoniana*	2014.12
JS02	Runzhou District, Zhenjiang City, Jiangsu Province	*P. massoniana*	2014.12
JS06	Binhu District, Wuxi City, Jiangsu Province	*P. massoniana*	2014.12
JS09	Xuyi County, Huai 'an City, Jiangsu Province	*P. massoniana*	2015.01
JS11	Haizhou District, Lianyungang City, Jiangsu Province	*Pinus densiflora*	2015.01
JS12	Yizheng, Yangzhou City, Jiangsu Province	*P. massoniana*	2015.01
JS15	Changshu City, Suzhou City, Jiangsu Province	*P. massoniana*	2015.02
JS19	Runzhou District, Zhenjiang City, Jiangsu Province	*P. massoniana*	2014.12
JS31	Lishui District, Nanjing City, Jiangsu Province	*P. massoniana*	2017.01
JS50	Jintan District, Changzhou City, Jiangsu Province	*P. massoniana*	2017.01

3.2. Statistics of SNP loci

The SNP locus information of 30 strains in Central China showed that there are 8,333,375 SNPs sites in total and the number of SNP sites varied significantly among different strains (Figure. 1). HB15, HEN20, HN07, HN08 and HN10 had significantly more SNPs and homozygotes than other strains. HEN15, HEN19, HEN20, HN09, HN10 and HN13 had significantly more missing SNPs than other strains. HEN20 had the highest number of SNPs, while HN02 had the lowest. HN10 had the highest number of homozygous, missing and private SNPs, which were 1033119, 5248382 and 505849, respectively.

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HN02 had the lowest number of homozygous SNPs, HEN06 had the lowest number of missing SNPs, and HN09 had the lowest number of private SNPs (Table 2 and Figure. 1).

Table 2. A summary of SNPs found in 30 *B. xylophilus* strains

Strains NO.	SNP count	Homozygous	Missing	Specific SNP count
HB01	81,723	46,927	1,367,096	6,644
HB02	110,859	75,239	1,189,888	6,547
HB03	128,236	34,233	957,159	2,695
HB05	90,192	45,461	1,318,256	2,291
HB06	91,540	57,049	965,177	2,290
HB07	121,555	69,319	1,038,514	9,634
HB08	85,121	33,889	1,277,353	2,195
HB10	230,409	66,327	954,380	7,499
HB12	156,058	92,696	978,007	8,018
HB15	747,217	620,622	1,364,706	10,220
HEN02	179,367	39,985	890,120	6,229
HEN03	115,598	73,932	1,032,402	13,361
HEN04	161,298	59,004	863,933	6,837
HEN06	387,158	74,788	670,407	31,052
HEN09	265,742	52,417	848,798	23,215
HEN10	215,398	41,954	816,941	6,011
HEN14	111,679	61,536	918,903	4,289
HEN15	125,089	47,713	5,169,332	1,586
HEN19	220,318	37,791	5,087,640	5,128
HEN20	748,317	620,041	5,223,813	3,968
HN01	123,168	86,716	946,562	1,316
HN02	38,277	14,055	1,572,950	716
HN03	124,574	91,984	928,412	1,906
HN04	58,334	25,275	1,484,882	2,413
HN06	373,296	26,536	1,482,689	80,083
HN07	894,621	704,750	747,078	24,547
HN08	878,428	611,007	837,408	12,192
HN09	130,567	23,176	5,151,805	396
HN10	1,210,980	1,033,119	5,248,382	505,849

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HN13	128,256	64,496	5,140,161	1,855

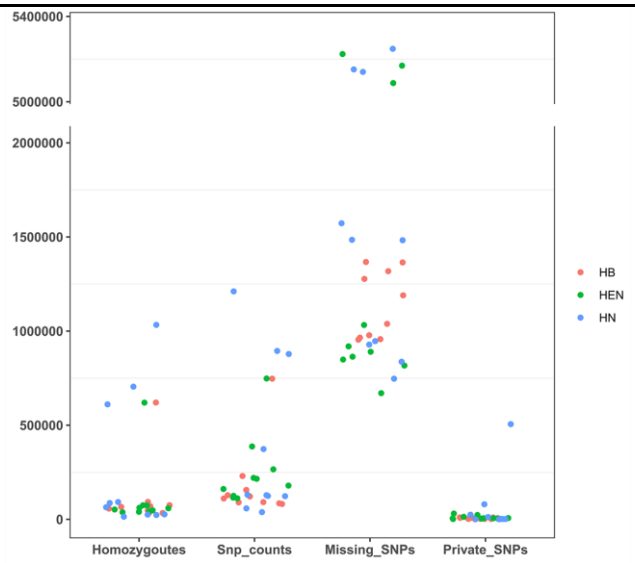

Figure 1. Homozygosity, SNP count, missing SNPs and private SNPs distributions of the SNPs found in 30 strains. Note: HB is Hubei strain, HEN is Henan strain, HN is Hunan strain. The same is below.

3.3. Statistics of SNP genotypes

The results of SNP genotyping on strains from Central China showed that there were 12 SNP genotypes: A>C, A>G, A>T, C>A, C>G, C>T, G>A, T>A, T>C and T>G. Comparing the genotype counts among strains, it is found significant differences for some genotypes. Specifically, four genotypes (A>G, C>T, G>A, and T>C) were significantly more frequent in HB15, HEN20, HN06, HN07, HN08, and HN10 strains than in other strains. For the remaining strains, six genotypes (A>G, C>G, C>T, G>A, G>C, T>C) were significantly more frequent than the others. Meanwhile, the genotype counts of HB15, HEN20, HN06, HN07, HN08 and HN10 strains were higher than those of other strains (Figure.2).

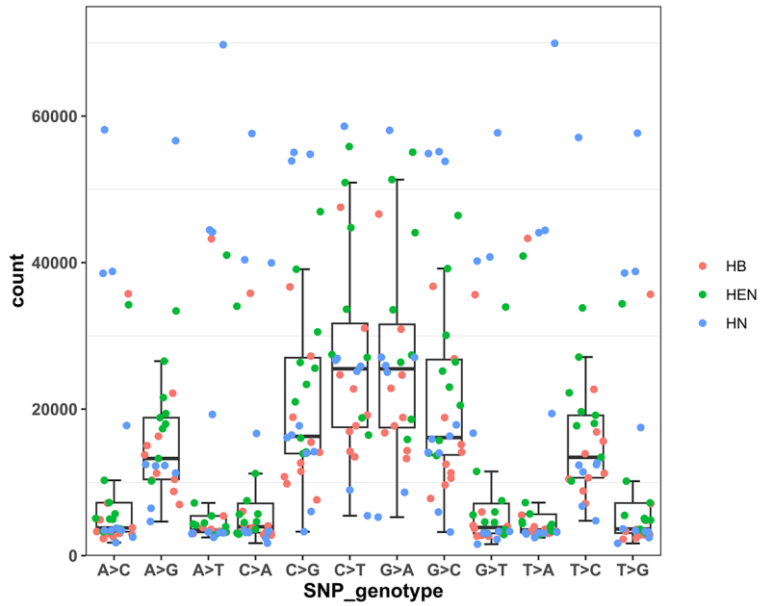

Figure 2. Box plots of SNP genotypes among 30 *B. xylophilus* strains.

3.4. Analysis of genetic differentiation

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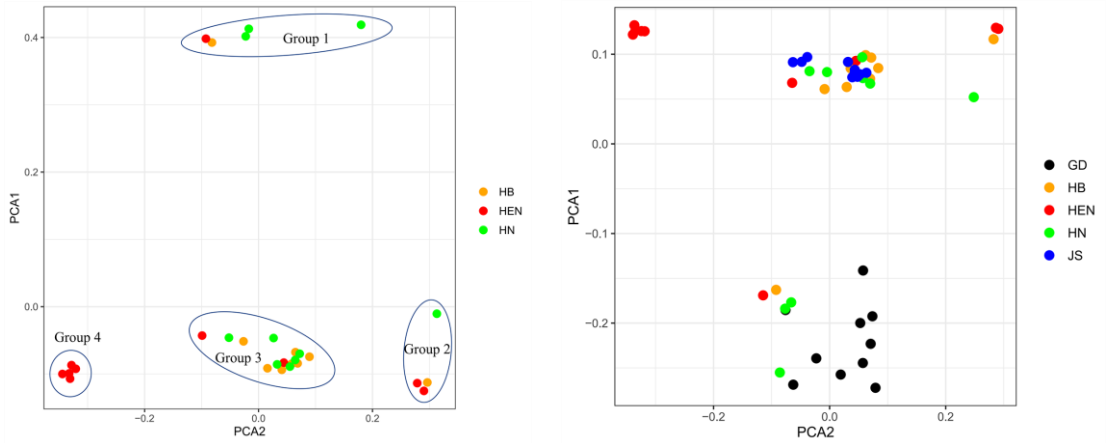
Principal component analysis (PCA) was conducted on 30 strains from Central China, which could be divided into four groups (Figure. 3a). Group 1 consisted of HB15, HEN20, HN07, HN08 and HN10. Group 2 comprised HB08, HEN02, HEN04 and HN06. Group 3 contained 16 strains, including 8 from Hubei, 2 from Henan and 6 from Hunan. Group 4 included only Henan strains: HEN06, HEN09, HEN10, HEN14 and HEN15. The PCA results revealed genetic differences among PWN populations in Central China. Henan Province had the highest genetic diversity, as its strains were distributed across all four groups. In contrast, most of the Hubei strains clustered in group 3, and the Hunan strains were either in group 1 or group 3.

To investigate the origin of PWN strains in Central China, the principal component analysis (PCA) was performed on the strains from Central China, Jiangsu Province, and Guangdong Province (Figure 3b). The PCA results indicated that the strains from Jiangsu Province were genetically similar to group 3 in Figure 3, while the strains from Guangdong Province clustered with group 1. The neighbor-joining tree of 50 PWNs confirmed the PCA results (Figure 4). Both analyses revealed that HB15, HEN20, HN07, HN08 and HN10 were closely related to strains from Guangdong Province, while the others were closely related to Jiangsu strains. Moreover, there were significant differences between Henan strains and strains from other provinces, suggesting different sources of invasion. To understand the invasion routes of PWN in central China, the introgression analysis was conducted and identified three possible pathways: 1) Guangdong to Henan; 2) Guangdong to Hunan; 3) Jiangsu to Hubei. This implies that Guangdong Province could be a major source of PWN spread in Henan and Hunan provinces, and Jiangsu Province could be a major source of PWN spread in Hubei Province (Figure 5).



(**a**) (**b**)

Figure 3. PCA of population genetic structure: (**a**) PCA results of 30 strains based on 1312 SNP markers; (**b**) PCA results of 50 strains based on 2244 SNP markers. Note: GD is Guangdong strain, JS is Jiangsu strain.

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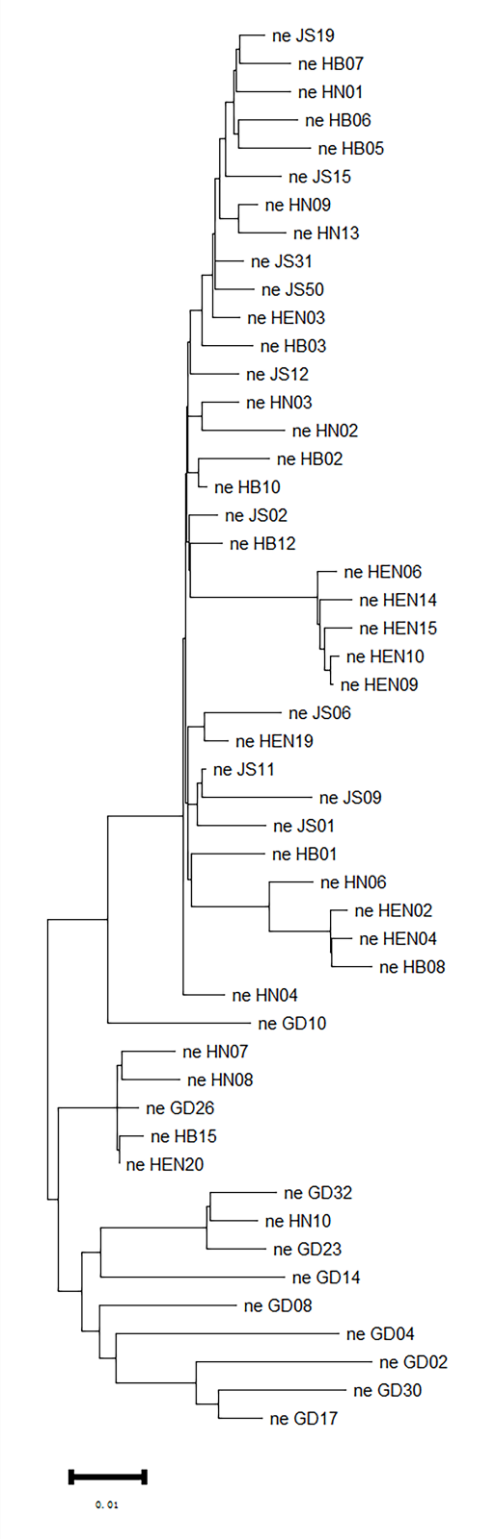

Figure 4. Phylogenetic trees of all 50 rains by based on Neighbor Joining method.

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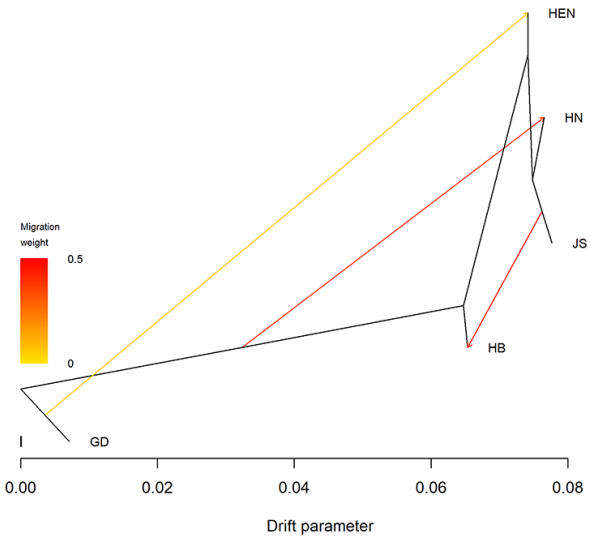

Figure 5. Introgression analysis revealed possible *B. xylophilus* migration routes.

4. Discussion

Previous studies have demonstrated that environmental factors can cause founder effects and genetic drift, leading to reduced or lost genetic diversity and population genetic differentiation in species that migrate and disperse [28-30]. Cheng et al. analyzed the genetic diversity of PWNs in different regions of China using AFLP markers and found that Chinese populations had slightly higher genetic diversity than American populations [31]. They found that Chinese populations were slightly higher than American populations in genetic diversity. However, Ding et al. used SNP markers to examine 181 PWN strains from 16 endemic areas in China and found that the Guangdong population had high genetic diversity and was genetically close to Americans, while the genetic diversity of strains in other areas tended to decrease [27]. They concluded that the invasive populations suffered from the loss of genetic diversity due to the founder effect, which was consistent with the findings of Mallez et al [29, 32]. These studies indicated that there was a specific correlation between different clusters and their geographical origin, and that SNP molecular marker technology was an effective tool to study the genetic differentiation of the PWN population [15, 23]. Based on the population structure analysis of PWNs in China [27], this study revealed the finer population structure in Central China for the first time using the SNP molecular markers.

The incidence and distribution of PWD in China were mainly concentrated in Guangdong and Jiangsu provinces, located in economically developed areas. Central China has a temperate and subtropical monsoon climate with an annual average temperature higher than 15℃, which is prone to PWD [33]. Moreover, the area is adjacent to Guangdong and Jiangsu provinces and has frequent trade activities with other parts of the country through traffic lines. It is possible that PWNs were introduced into infected pine plants and their products (cable trays, packing cases, etc.) during trade activities. Therefore, PWNs in each epidemic area of central China may have different epidemic sources, and there is a high possibility of cross-invasion. This is also consistent with our research results that there are multiple clusters in central China.

This study applied SNP molecular marker technology to analyze the genetic differences of PWN populations in central China. The results revealed that the number of SNP sites, homozygote number, and genotypes of HB15, HEN20, HN07, HN08 and HN10 were significantly higher than those of other strains, and the number of missing SNP sites differed significantly from other strains. The results showed that these strains had distinct SNP loci and genotypes, which was consistent with the clustering results. The clustering

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results showed that the five strains were genetically distant from other strains. Therefore, we inferred that there were different sources of transmission for the strains in Central China. Ding[27] analyzed the population genetic structure of strains from different regions of China, and the results showed that the strains from Henan, Hubei and Hunan were clustered into one group, among which the Hunan strains were distributed in several groups, showing rich genetic diversity. It was also confirmed that the insect strains in central China had different transmission sources.

Previous studies suggested that Guangdong Province was the initial colonization and dispersal center of pine wood nematode in China, and Jiangsu Province was a new dispersal center. Therefore, this study analyzed the population genetic structure of the strains from central China and those from Jiangsu and Guangdong Province. The PCA and phylogenetic tree demonstrated that HB15, HEN20, HN07, HN08 and HN10 were closely related to the strains from Guangdong Province, while other strains were closely related to those from Jiangsu Province. The introgression results also confirmed that the Henan and Hunan isolates originated from Guangdong strains. The result may be due to the genetic difference between Henan and Hubei populations in the isolated taxa. Therefore, we hypothesized that there were three main transmission routes for the strains from Central China (Guangdong to Henan; Guangdong to Hunan; Jiangsu to Hubei), and that HB15, HEN20, HN07, HN08 and HN10 strains were invasion from Guangdong. This was consistent with Ding's results[27], indicating that the Hunan strains migrated from the Guangdong strains. This study further analyzed population genetic diversity in Central China. There was evidence of multiple invasions and cross-invasions in the epidemic areas of Hubei, Henan and Hunan, which indicated that the regulation of infected wood was insufficient.

Wang et al. used SNP to analyze the genetic differentiation of PWN populations in East China [22] and found correlations among different groups and geographical regions. However, the genetic differentiation within each group was not significant. In contrast, this study revealed that there was genetic differentiation of PWNs from central China, and the genetic diversity of Henan strains was higher. Some Henan strains were not only genetically differentiated from those in Guangdong province but also had genetic distance from those in Jiangsu province, which might result from genetic drift and founder effect during the invasion of PWNs. Huang et al. reported significant genetic differences among PWN populations in Guangdong Province [13]. Ding et al. suggested that the Guangdong strain had similar genetic variation to the American strain and speculated that it might originate from foreign invasion [15, 27, 34]. We hypothesized that the Henan strain might also be influenced by foreign invasion because it had a distinct genetic structure. However, there was a lack of a large number of foreign strains to verify this possibility.

5. Conclusions

By analyzing the genetic diversity of PWNs from central China, we found that some strains had higher genetic similarity to those from Guangdong. In addition, the Henan strain has rich genetic diversity and genetic differences from other strains, suggesting that there may be different transmission sources. The results provide the theoretical basis for exploring the propagation path of pine wood nematode, population genetic structure and formulating a quarantine strategy.

Author Contributions: A.-X.Y.: designed the study, conducted the experiment, performed the data analysis and wrote the article; X.-L.D.: guided the article writing and data analysis; Y.F. and T.-T. C.: collected the samples; J.-R.Y.: guaranteed the integrity of the entire study and approved the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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