



Investigation of beetle species that carry the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhner) Nickle, in China

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Received: 17 October 2019 / Accepted: 29 December 2019 / Published online: 18 May 2020
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Abstract In order to found new carriers of pine wood nematode (PWN), *Bursaphelenchus xylophilus*, beetles were collected from pine wilt disease-affected areas in six provinces in China. A total of 8830 beetles of 29 species was collected and examined to determine whether they were PWN carriers. Eight species were identified as carriers. Results included the first worldwide report of *Monochamus nigromaculatus*, *Semanotus sinoauster*, and *Uraecha angusta* being carriers of PWN, and the first report from China of *Arhopalus rusticus* carrying PWN. *Monochamus alternatus* was commonly collected in all six provinces and was the dominant species in four inland affected areas and *A. rusticus* was dominant in two coastal affected areas. The species varied between different neighboring regions in the same province. The

distribution of the same species varied considerably over different regions.

Keywords Beetle · Carrier · Pine wilt disease · Pine wood nematode · Vector

Introduction

Pine wilt disease (PWD), caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhner) Nickle, is the most destructive disease affecting pine species in China and causes significant economic losses (Yang et al. 2003). PWD is regarded as an “incurable disease” because infected pines die rapidly and there are no effective measures available for its treatment. PWN is native to North America where it usually damages only exotic (i.e., introduced non-native) pine species, but it has also spread to countries in Asia such as Japan, China and South Korea, and to European countries of Portugal, and Spain (Zhao et al. 2008; Vicente et al. 2012). PWD spreads rapidly and is extremely difficult to prevent and control. Therefore, PWD has been listed as a quarantine disease in many countries. Studies have suggested that at least 45 insect species may carry PWN, belonging to the families Cerambycidae, Buprestidae, Curculionidae, Scolytidae, and Termitidae (Zhang et al. 2007). However, not all insect species that carry PWN can transmit it; only ones with life histories matching that of PWN may become vectors. The activities of vector insects, especially feeding and oviposition behaviors, are the basic modes responsible for the natural transmission of PWN (Togashi and Shigesada 2006). Of the 45 insect species known to carry PWN, only 13 act as vectors. These 13 insect species all belong to the family of long-horned beetles, *Monochamus* spp. (Cerambycidae) (Zhang et al. 2007). The regional

Project funding: The work was supported by the National Key Research and Development Program of China (Grant Number: 2017YFD0600104), and the Shenyang Science and Technology Planning Project (Grant Number:18-400-3-03).

The online version is available at <http://www.springerlink.com>.

Corresponding editor: Tao Xu.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11676-020-01146-2>) contains supplementary material, which is available to authorized users.

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distribution of these 13 beetle species are *Monochamus alternatus* Hope, *Monochamus saltuarius* Gebler, *Monochamus grandis* Waterhouse, and *Monochamus sutor* Linnaeus in Asia, and *Monochamus galloprovincialis* Olivier and *M. sutor* in Europe, with the remaining eight species common to North America (Sousa et al. 2001; Zhang et al. 2007). Vector beetles that spread PWN are often dominant species in the local area, e.g., the main vector of PWN in Asia is *M. alternatus* (Chai and Jang 2003), in North America, it is *Monochamus carolinensis* Olivier (Linit et al. 1983; Chai and Jang 2003), and in Europe, it is *M. galloprovincialis* (Sousa et al. 2001). *Monochamus* spp. beetles are recognized as the most important vectors of PWN (Kim et al. 2003; Ning et al. 2004). *M. alternatus* (Chai and Jang 2003) and *M. saltuarius* (Yu and Wu 2018) are the known vectors of PWN in China. Previous studies have also reported potential PWN vector beetles. *Acanthocinus griseus* Fabricius, *A. gundaiensis* Kano (Xu et al. 1993), *Arhopalus rusticus* Linnaeus (Linit et al. 1983), *Aromia bungii* Faldermann (Wang et al. 1985) have been reported to be carriers of PWN in China. *Asemum striatum* Linnaeus, *Corymbia succedanea* Lewis, *Spondylis buprestoides* Linnaeus, *Uraecha bimaculata* Thomson have been identified as PWN carriers in Japan (Zhang et al. 2007), with these as well as the species of beetle in China. Numerous studies have investigated the beetle vectors of PWN but these studies may not have been thorough and it is possible that some carriers have not been identified and may be potential PWN vectors. To better understand the species of beetles that carry PWN in China, beetles were collected from PWD-affected areas and identified the presence of PWN by real-time polymerase chain

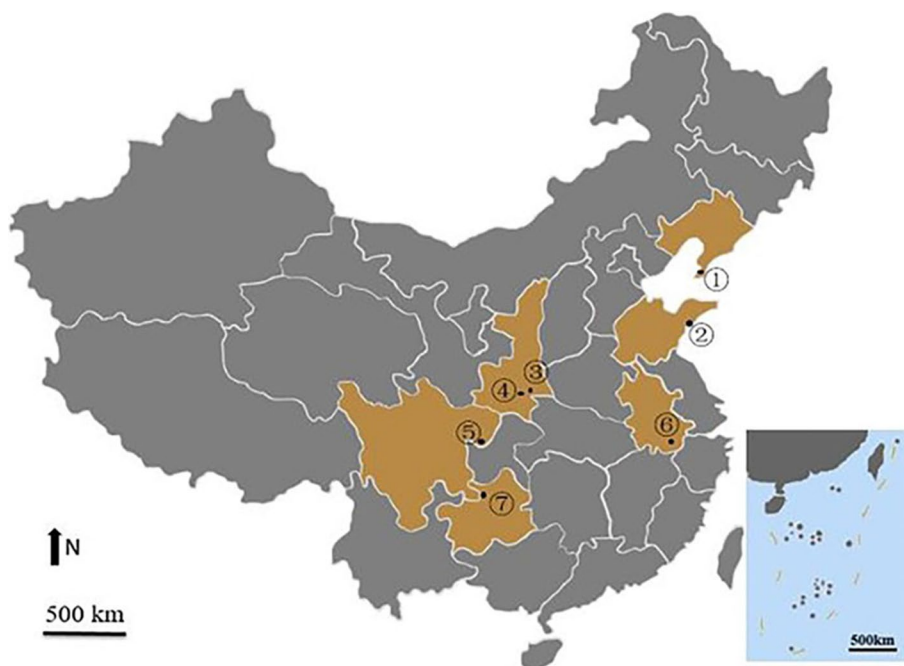
reaction (PCR), also known as quantitative polymerase chain reaction (qPCR) (Chen et al. 2007). Identifying the beetle species that carry PWN is important for reducing or limiting the spread of PWN and for improving the level of control. Thus, the results of this study should provide a theoretical basis for the control of PWD.

Materials and methods

Between May and September 2019, beetles were collected from PWD-affected areas in Dalian, Liaoning Province, Linshui, Sichuan Province, Huangshan, Anhui Province, Zhashui and Foping in Shaanxi Province, Qingdao, Shandong Province and Renhuai, Guizhou Province (Fig. 1). Ten traps (Supplementary: Fig. S1) with APF-I lure (CHEN KAN AGROFORESTRY, Xiamen, China) used to trap beetles were hung in each affected area. Numbers and species were recorded every three days. Photographs of the beetles were taken with a Zeiss stereoscopic microscope (StEREO Discovery.V20, Carl Zeiss, Göttingen, Germany).

Each beetle was placed in a surface-sterilized mortar, frozen in liquid nitrogen, ground to a powder and transferred to a 2-mL Eppendorf tube with 1.5 mL of nematode lysis solution (Zhang et al. 2017) and 5 μ L of protease K (Takara, Dalian, China). The mixture was then subjected to centrifugation at 2000 \times g for 1 min before heating for 45 min at 65 $^{\circ}$ C in a constant temperature water bath, with the temperature then being raised to 95 $^{\circ}$ C for 10 min. Samples were then centrifuged at 12,000 \times g for 3 min, before collecting the supernatant and storing it at 4 $^{\circ}$ C.

Fig. 1 Beetle sampling locations. ① Dalian (121° 61' E, 38° 88' N), Liaoning Province; ② Qingdao (120° 51' E, 36° 24' N), Shandong Province; ③ Zhashui (109° 13' E, 33° 61' N), Shaanxi Province; ④ Foping (107° 98' E, 33° 73' N), Shaanxi Province; ⑤ Linshui (107° 01' E, 30° 06' N), Sichuan Province; ⑥ Huangshan (118° 22' E, 29° 66' N), Anhui Province; ⑦ Renhuai (106° 36' E, 27° 85' N), Guizhou Province



The 10- μ L detection mixture consisted of 3.5 μ L of the supernatant, 5 μ L of Premix Ex Taq (Takara, Dalian, China), and 1.5 μ L of a mixture of probe (5'-TGCAC GTTGT GACAG TCGT-3') and primers (F: 5'-GAGCA GAAAC GCCGA CTT-3', R: 5'-CGTAA AACAG ATGGT GCCTA-3') (GAQSIQ 2017) in the PCR tube which was centrifuged at 2000 \times g for 30 s. The detection mixture was analyzed with LineGene K Plus Real-Time PCR Detection System (FQD-48A, Hangzhou Bioer Technology Co. Ltd. Hangzhou, China) for PWN (constant temperature: 95 °C for 20 s; 40 cycles at 95 °C for 15 s, and 60 °C for 20 s).

Results

There were 8830 beetles belonging to 29 species collected from the PWD-affected areas in the six provinces (Table 1).

Twelve species were collected in Qingdao, the most frequent were *A. rusticus*, which accounting for 72% of the total, and *M. alternatus*, accounting for 25%. Ten species were collected in Dalian. Of these, there were 388 specimens (65%) of *A. rusticus*, 105 (18%) of *M. alternatus* and 51 (8.6%) of *M. saltuarius*, making them the most frequent beetle species in this PWD-affected area. In Huangshan, nine species were collected, the major ones were *M. alternatus*, accounting for 85% and *A. griseus*, accounting for 12%. Seventeen species were collected in Shaanxi Province (Foping and Zhashui), eleven in Zhashui, where *M.*

Table 1 Species of beetle collected in pine wilt disease-affected areas in six provinces

Species	Number of beetles collected						
	SD	LN	AH	SX		SC	GZ
				Foping	Zhashui		
<i>Calolepta permutans</i> Pascoe				5			
<i>Acalolepta sublusca</i> Thomson	2					4	
<i>Acanthocinus griseus</i> Fabricius		6	164		12	32	48
<i>Anoplophora chinensis</i> Forster	5	9	7		1		3
<i>Apriona rugicollis</i> Chevrolat	8	4					5
<i>Arhopalus rusticus</i> Linnaeus	3040	388	16		9	15	12
<i>Aristobia hispida</i> Saunders				1			
<i>Aromia bungii</i> Faldermann	20	12	2				
<i>Chloridolum thaliodes</i> Bates							1
<i>Dorysthenes granulosus</i> Thomson	1						
<i>Lamiomimus gottschei</i> Kolbe		5			3		2
<i>Megopis sinica</i> White	12	11					7
<i>Mesosa myops</i> Dalmsn				3			
<i>Monochamus alternatus</i> Hope	1073	105	1138	618	865	371	208
<i>Monochamus nigromaculatus</i> Gressitt					323		
<i>Monochamus saltuarius</i> Gebler		51					
<i>Monochamus sparsutus</i> Fairmaire			2		3		
<i>Olenecamptus cretaceus marginatus</i> Schwarzer			3	6			
<i>Paraleprodera diophthalma</i> Pascoe				2	9		
<i>Pogonocherus dimidiatus</i> Blessig							8
<i>Purpuricenus petasifer</i> Fairm		2			1		
<i>Purpuricenus temminckii</i> Guerin							2
<i>Spondylis buprestoides</i> Linnaeus			5	7			
<i>Semanotus sinoauster</i> Gressitt	70					17	
<i>Trirachys orientalis</i> Hope	1						
<i>Uraecha angusta</i> Pascoe			2	3	52		
<i>Uraecha yunnana</i> Breuning				5			
<i>Xylotrechus magnicollis</i> Fairmaire	1				1		
<i>Xylotrechus quadripes</i> Chevrolat	1						
Total number	4234	593	1339	650	1279	450	285

SD: Qingdao, Shandong Province; LN: Dalian, Liaoning Province; AH: Huangshan, Anhui Province; SX: Shaanxi Province; SC: Linshui, Sichuan Province; GZ: Renhuai, Guizhou Province

alternatus accounting for 68% of the total and *Monochamus nigromaculatus* for 25%. Nine species were collected in Foping with *M. alternatus* accounting for 95%. Only three species (*M. alternatus*, *Uraecha angusta*, and *Paraleprodera diophthalma*) were found in each of Zhashui and Foping. Eight species were collected in Linshui, with *M. alternatus* the most abundant, accounting for 82%. Seven species were collected in Renhuai, with *M. alternatus* the most abundant, accounting for 73%, followed by *A. griseus* with 48 specimens (17%).

In this study, all the beetle species were examined to determine whether they were PWN carriers. The results are shown in Table 2, in which eight (supplementary Figs. S2–S9) were carriers, namely *Aromia bungii*, *A. griseus*, *A. rusticus*, *M. alternatus*, *M. nigromaculatus*, *M. saltuarius*, *U. angusta*, and *Semanotus sinoauster*. For the first time, *M. nigromaculatus*, *S. sinoauster*, and *U. angusta* were

identified as PWN carriers (qPCR results: supplementary Figs. S10–S12), and for the first time in China, *A. rusticus* was identified as a PWN carrier (qPCR results: supplementary Fig. S13).

Discussion

This study is the first worldwide report of PWN detected in *M. nigromaculatus*, *S. sinoauster* and *U. angusta*. *M. nigromaculatus* is a member of *Monochamus* spp. (Cerambycidae), to which all previously identified PWN vectors belong (Zhang et al. 2007). Therefore, it is very likely that *M. nigromaculatus* is a vector insect. It is also one of the most important beetle species in the sampling area, accounting for 25% of the total number collected. Therefore, it should be further studied with the ultimate aim of improving

Table 2 Beetle species identified as pine wood nematode (PWN) carriers

Species of beetles	Number of beetles examined	Number of beetles carrying PWN	Percentage (%) ^a
<i>Acalolepta permutans</i>	5	0	0
<i>Acalolepta sublusca</i>	6	0	0
<i>Acanthocinus griseus</i>	30	4	13
<i>Anoplophora chinensis</i>	25	0	0
<i>Apriona rugicollis</i>	17	0	0
<i>Arhopalus rusticus*</i>	30	6	20
<i>Aromia bungii</i>	30	3	10
<i>Aristobia hispida</i>	1	0	0
<i>Chloridolum thaliodes</i>	1	0	0
<i>Dorysthenes granulosus</i>	1	0	0
<i>Lamiomimus gottschei</i>	10	0	0
<i>Megopsis sinica</i>	30	0	0
<i>Mesosa myops</i>	3	0	0
<i>Monochamus alternatus</i>	30	9	30
<i>Monochamus nigromaculatus</i> ^b	30	6	20
<i>Monochamus saltuarius</i>	30	7	23
<i>Monochamus sparsutus</i>	5	0	0
<i>Olenecamptus cretaceus marginatus</i>	9	0	0
<i>Paraleprodera diophthalma</i>	11	0	0
<i>Pogonocherus dimidiatus</i>	8	0	0
<i>Purpuricenus petasifer</i>	3	0	0
<i>Purpuricenus temminckii</i>	2	0	0
<i>Semanotus sinoauster</i> ^b	30	3	10
<i>Spondylis buprestoides</i>	12	0	0
<i>Trirachys orientalis</i>	1	0	0
<i>Uraecha angusta</i> ^b	30	5	17
<i>Uraecha yunnana</i>	5	0	0
<i>Xylotrechus magnicollis</i>	2	0	0
<i>Xylotrechus quadripes</i>	1	0	0

^aPercentage carrying PWN to the total examined

^bNewly discovered species that carry PWN

the prevention and control of PWD in that area. However, very few studies have investigated *M. nigromaculatus*, so it is necessary to study its habits and determine whether it can transmit PWN. In addition, *U. angusta* belong to *Uraecha* spp. (Cerambycidae) and *S. sinoauster* to *Semanotus* spp. (Cerambycidae) (Hua et al. 2009). Although these two species do not belong to the *Monochamus* genus, their host plants include members of the Pinaceae (Hua et al. 2009), and therefore it would be important to determine whether these two potential vectors can transmit PWN.

Monochamus alternatus, the Japanese pine sawyer, is the main vector responsible for the transmission of the pine wood nematode in China (Ning et al. 2004), and thus it is the main target vector for the prevention and control of PWD. In this study, analysis showed that the distribution of *M. alternatus* was very wide, occurring across all PWD-affected areas in the six provinces, and it accounted for high percentages of the specimens collected. *M. alternatus* was dominant among the species collected in the PWD-affected areas in Anhui (85%), Sichuan (82%) and Guizhou (73%) and Shannxi provinces (71%), reflecting to some extent the severity of PWD in these areas. However, the proportion of *M. alternatus* among the species collected in Liaoning (18%) and Shandong provinces (25%) were low. The relative distribution of *M. alternatus* in China is more towards the south and less towards the north (Table 1). A previous study had reported that cold stress may influence the persistence of populations of *M. alternatus* in regions north of 30° N and south of 30° S, so the effective accumulated temperature might limit the spread of *M. alternatus* (Song and Xu 2006).

Arhopalus rusticus is a highly devastating trunk-boring longhorn beetle that causes considerable damage to trees, particularly members of the Pinaceae and Taxodiaceae families, *Cupressus* spp. and *Quercus* spp., as well as to the *Alnus cremastogyne* Burk. (Bradbury 1998; Wang et al. 2016). Zhao et al. (2004) noted that *A. rusticus* is the most damaging trunk-boring insect that carries of *Bursaphelenchus mucronatus*, a nematode species closely related *B. xylophilus*, but it is not clear whether it can carry *B. xylophilus*. However, this study showed that 20% of *A. rusticus* specimens from PWD-affected area carried PWN. This result is similar to that reported in previous studies that suggested that *A. rusticus* can carry PWN (Linit et al. 1983) and is a potential vector of PWN (Jurc et al. 2012). Previous studies extracted PWN from *A. rusticus* and that it might spread PWN (Mamiya and Enda 1972; Ridley et al. 2001). In this study, *A. rusticus* was identified for the first time in China as a PWN carrier. *A. rusticus* specimens were collected from all six provinces and it was the major beetle species in Liaoning and Shandong Provinces. However, the numbers in the other four provinces were lower, possibly due to different environments, with low numbers in inland areas and higher numbers in coastal areas. Whether this phenomenon is found

everywhere remains to be further studied. It is not known if *A. rusticus* transmits PWN, but further research is merited because of the high abundance of this beetle. Additional studies should focus on its habits.

Acanthocinus griseus is a carrier of PWN in Japan and China (Kobayashi et al. 1971; Xu et al. 1993), and host plants include *Pinus koraiensis* Sieb., *Pinus tabuliformis* Carr., *Picea jezoensis* Carr., *Pinus armandii* Franch., *Pinus massoniana* Lamb., *Picea asperata* Mast., *Juglans regia* L.; *Quercus* spp. and *Populus* spp. (Hua et al. 2009). In this study, *Arhopalus griseus* specimens were collected from five provinces and it was one of the main beetle species in the Anhui PWD-affected area. Therefore, it is necessary to study the habits of this beetle to determine whether it can transmit PWN. *M. saltuarius* is an important vector of PWN in Japan (Kobayashi et al. 1984) and Korea (Han et al. 2007) and a recent study found that it can transmit PWN in China (Yu and Wu 2018). Wang et al. (1985) collected an individual of *A. bungii* from a *Pinus thunbergii* Parl. log and this beetle carried 275 individual PWNs. In this study, 13% of *A. bungii* examined carried PWN. Members of the Pinaceae are not included among the host plants of *A. bungii* (Hua et al. 2009) and did not feed on Pinaceae in the laboratory. Whether *A. bungii* will oviposition in Pinaceae, emerge and carry pine wood nematodes needs to be further studied. Other beetles that can carry PWN are from the Lamiinae family, but *A. bungii* and *S. sinoauster* are from the Cerambycinae and *A. rusticus* from the Aseminae. Therefore, beetles belonging to Cerambycinae and Aseminae should be evaluated.

It has been found that latitude has a considerable impact on the distribution of biological species (Stevens 1989). In this study, the overall species, number and the major species collected varied greatly among the six provinces. In addition, the species collected varied among different neighboring regions. Seventeen different beetle species were detected in the PWD-affected areas in Zhashui and Foping, (only approximately 100 km apart), in Shaanxi Province, but only *M. alternatus*, *P. diophthalma*, and *U. angusta* were found in both areas. In spite of their proximity, environmental differences presumably resulted in the differences in beetle species present in the two regions. Both are in the Qinling Mountains with complex topography and climatic environments. In addition, there were differences in distribution numbers of the same species in different regions (Song and Xu 2006). For example, the abundance of *A. rusticus* was low in inland areas but high along the coast, whereas *M. alternatus* had the opposite distribution pattern. Studies have shown that climate is one of the main factors that restricts the distribution, growth, and reproduction of living organisms (Parmesan and Yohe 2003). Moreover, increasing evidence suggests that temperature increases, changes in precipitation patterns, and other climate extremes may have major impacts on biodiversity (Parmesan and Yohe 2003;

Root et al. 2003). Thus, climate differences in coastal and inland areas, as well as changes in latitude from south to north, may explain the different beetle distributions identified in this study.

Only PWD-affected areas in some provinces were investigated in this study and it was not comprehensive in terms of beetle species collected and examined for PWN. In future research, the scope of this research will be expanded in order to track and investigate the dynamic changes in beetle populations in PWD-affected areas, as well as to determine the life habits of the major beetle species and their relationships with and ability to transmit PWN.

Conclusions

Monochamus nigromaculatus, *S. sinoauster*, and *U. angusta* were identified as pine wood nematode (PWN) carriers for the first time worldwide, and, for the first time in China, *A. rusticus* was identified as a PWN carrier. The species, number and major species of beetle collected varied considerably among the various PWD-affected areas in this study. The species collected varied among different neighboring regions and there were differences in distributions of the same species among different regions.

Acknowledgements The authors acknowledge the support of the Forestry Bureaus of Dalian, Qingdao, Huangshan, Renhuai, Linshui, Zhashui and Foping. The authors are grateful to the Area Editor and reviewers whose comments have contributed to improving the quality of this paper.

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