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Detecting Non-Local Japanese Pine Sawyers in Yunnan, Southwestern China via Modern Molecular Techniques

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1. Introduction

The term “biological invasion” comes with two aspects, on one hand it refers to the introduction of an exotic species, and/or non-local populations of any species to a given geographical area, while on the other hand it refers to the ecological and/or the economical consequences of such activity (Perrings et al., 2002). With accelerating socioeconomic development and globalization, issues of biological invasion are of increasing concern, as some invasive alien species are capable of causing catastrophe to local environments and the economies (Xu et al., 2006; Meyerson & Mooney, 2007).

Intentionally or accidentally, human beings constantly introduce organisms to new habitats. For those cases of intentional introduction, some were for agronomic purposes and have been proved to be beneficiary, such as potatoes, maize, peanuts, and sunflowers (Pope et al., 2001; S.M. Wang, 2004). However, others like *Eichharnia crassipes* (Martius) had caused a diversity of problems (Harley et al., 1996; Julien, 2001; T.J. Hu et al., 2009). For accidental introduction, the consequences are often more negative, as these organisms often go undetected until they have expanded their population considerably. The invasion and expansion of *Eupatorium adenophorum* Sprengel in western China is a typical example (R. Wang & Y.Z. Wang, 2006; R. Wang et al., 2011).

Yunnan Province in southwestern China is situated in the low-latitudinal plateau region (LLPR) connecting the Indochinese peninsula and the western portion of mainland China, where the terrain and climate are complex (Z.Y. Chen, 2001; S.Y. Wang & W. Zhang, 2005). Such distinct geographical position and natural environment entitle Yunnan a wide scope of ecosystems except desert and ocean in the northern hemisphere (Guo & Long, 1998). Apart from being the representative of terrestrial biodiversity, such complexity also makes Yunnan very vulnerable to invasive alien species.

The western portion of Yunnan is generally known as the longitudinal range-gorge region (LRGR) where great mountains and deep valleys run parallel through Yunnan and connect the Indochinese peninsula and the inner area of western China, which has been considered as the terrestrial pathway of biological invasion in Yunnan (He et al., 2005). The eastern

portion of Yunnan is the western margin of the Yunnan-Guizhou altiplano, where most of the terrain are plains with low hills with an altitude lower than the central portion of Yunnan as well as most of the LRGR (S.Y. Wang & W. Zhang, 2005). The subdued slope of this area also allows non-local organisms to enter Yunnan from the adjacent areas of China. Meanwhile, Yunnan also plays an important role in preventing invasive alien species from entering China, as the mountains lie in northern portion are natural barriers to obstruct the populations of non-local organisms from expanding. Therefore, Yunnan bears a vital strategic function of regional ecosecurity defined by the combination of terrestrial pathway and frontier prevention of biological invasion.

To enhance relevant research in detecting exotic species and/or non-local populations in Yunnan, research on invasive alien species has been conducted to reveal origin, expansion, and the mechanisms of invasion. For cases of invasive insects, researches on two exotic tephritid pests, *Bactrocera dorsalis* (Hendel) and *B. correcta* (Bezzi) have achieved the goals of population recognition and route reestablishment via modern molecular techniques (Shi et al., 2010; Liu, unpublished data). These studies demonstrated that the invasion of the two fruit flies represented the mode of natural and long-term invasion, inhabitation, and expansion. Moreover, the merits of these important research also provided ideas for the research on biological invasion in the new era. We used molecular techniques to detect non-local populations of a forest pest in southwestern Yunnan which strongly suggested another possible mode of biological invasion.

2. Background information

2.1 The Japanese pine sawyer

The Japanese pine sawyer, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), is a stem-boring beetle widely distributed in eastern Asia and the northern portion of the Indochinese peninsula (Davis et al., 2008). This polyphagous beetle feeds on conifers throughout the life history, including many unrelated species of *Pinus*, *Picea*, *Abies*, *Cedrus*, *Larix*, and *Cupressus* (Ning et al., 2004; Davis et al., 2008). *M. alternatus* is a univoltine species, which produces only one generation per year (Togashi, 1989; L.P. Wang, 2004; Zhao et al., 2004). After copulation, the female adults gnaw ovipositing wounds in the bark of host trees, and lay eggs in the space between the phloem and the xylem (Anbutsu & Togashi, 2000). The newly hatched larvae ingest wood tissue from both the phloem and the xylem, and start to excavate "U" shaped tunnels from the third instar. The final instar larvae build oval pupal chambers at the end of the tunnels to pupate. The newly eclosed adults feed on shoots, needles, and bark to obtain nutrients for maturation (Shibata, 1987). Copulation usually takes place five to ten days after emergence, each adult is able to mate more than once (L.P. Wang, 2004; H. Yang et al., 2006). As a typical secondary stem-boring species, the female adults tend to select stressed hosts for oviposition (S.J. Hu et al., 2009), which is induced by the volatile chemicals (i.e., α -pinene, β -pinene, 3-carene, and ethanol) emitted from the hosts (Ikeda et al., 1986; Yamasaki et al., 1989), but often influenced and deterred by bark thickness, branch diameter, ovipositing scars from other female adults, and larval frass (Nakamura et al., 1995a, 1995b; Anbutsu & Togashi, 2000; Li & Z.N. Zhang, 2006; S.J. Hu et al., 2009; Z.X. Yang et al., 2010).

2.2 Association with the pine wood nematodes

The pine wood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhner) (Nematoda: Aphelenchoididae), is a quarantine phytopathogenic organism of conifers indigenous to northern America but casually spread to Eurasia and southern Japan in last century (CABI & EPPO, 1997). The nematode, like other species of genus *Bursaphelenchus*, lives in the vascular tissue of their coniferous hosts, which decreases the transportation of water and resin, and subsequently weakens the hosts and causes a syndrome named the pine wilt disease (Mamiya, 1983). The pathogenicity of *B. xylophilus* varies significantly. In North America, reports on its damages are rarely seen (Wingfield et al., 1986). However, in the vast majority of Japan and China, where *B. xylophilus* is considered as an invasive species, the infestation is often fatal, and has caused catastrophic timber loss (Mamiya, 1988; X.B. Hu et al., 1997). In nature, the relocation of *Bursaphelenchus* nematodes depends on coleopterous vectors, but the association between nematodes and vectors differs with taxa (Linit, 1988). For *B. xylophilus*, the long-horned beetles of genus *Monochamus* are the major vector (Linit et al., 1983; Evans et al., 1996; Kulinich & Orlinskii, 1998), and research has confirmed that *M. alternatus* is the key vector of *B. xylophilus* in eastern Asia (Mamiya & Enda, 1972; Kobayashi et al., 1984). In infested hosts, the immature pine wood nematodes are able to locate the pupae of *M. alternatus* and board into the tracheae, and the newly eclosed adults carry the nematodes when seeking food and new hosts. During the course of feeding and ovipositing, the nematodes detach from the beetles and enter the new hosts through the feeding and ovipositing wounds to initiate a new round of infestation (Edwards & Linit, 1992; Naves et al., 2007).

2.3 Economic importance of *M. alternatus*

There are basically four aspects of economic importance of *M. alternatus*. As a stem-boring coleopteran of the conifers, the direct feeding and ovipositing of the adults damages the tender shoots, needles, and bark of the host, which may weaken the host when a sizable population occurs in a stand. The tunnels inside the host built by the larvae often cause more damage which eventually kills the host and substantially reduces the economic value of the timber (L.P. Wang, 2004). *M. alternatus* spends the entire immature stage in the host, which makes it very difficult to be detected from timber and/or wood packaging materials and can be easily relocated to new habitats via transportation in trading (Haack, 2006). As timber and/or wood packaging materials with *B. xylophilus* can also be relocated (Braasch et al., 2001; Gu et al., 2006), the concealed *M. alternatus* would subsequently introduce the nematodes into new habitats via its dispersal. Hence, *M. alternatus* has been recognized as a dangerous forest pest by forestry authorities worldwide.

2.4 The pine wilt disease in China

In mainland China, the pine wilt disease was reported for the first time in a small patch of *Pinus thunbergii* near the Sun Yat-sen Mausoleum in Nanjing, Jiangsu Province in 1982 (Y.Y. Wang et al., 1991). Afterwards, the infested area expanded quickly throughout eastern China. From the bulletins released by the SFA (State Forestry Administration) of China, a clear tendency of westward expansion can be observed (SFA, 2004 ~ 2007). During the past two years, some counties in Henan, Shaanxi, and Sichuan provinces in northern, northwestern, and southwestern China also reported infested areas of the pine wilt disease (SFA, 2011) (Fig. 1).

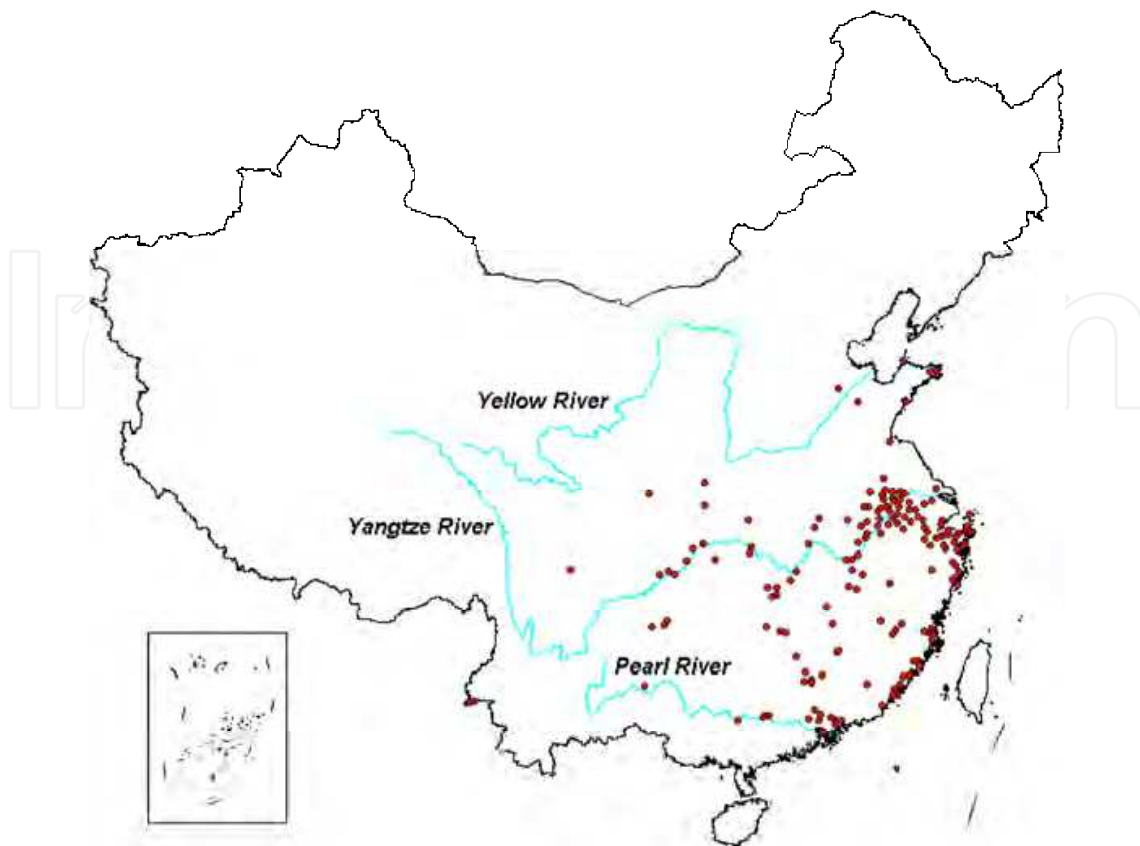


Fig. 1. The distribution pattern of the pine wilt disease (red dots) in mainland China based on the historical data from 2004 ~ 2011, data source: SFA (2004 ~ 2011).

With the expansion of pine wilt disease, an interesting distribution pattern was revealed. In eastern and southern China, the distribution concentrated near harbors, like the deltas of the Yangtze River and the Pearl River, while in the central portion of China, the infested areas are mostly along the Yangtze River. Such a distribution pattern suggested that the dispersal of *B. xylophilus* was closely related to transportation.

The occurrence of the pine wilt disease in Yunnan Province, southwestern China was distinct. Not only because it was one of the latest infested areas after the massive expansion, but also due to its unique geographical position. Unlike eastern China where many harbors are distributed, the first, single, and isolated pine stand (*Pinus kesiya* var. *langbianensis*) in Yunnan infested by *B. xylophilus* was located in the westernmost corner, a remote township named Wanding near the Sino-Burmese border (SFA, 2007).

3. The hypothesis

Given that there had been no report on the occurrence of *B. xylophilus* in Burma (CABI & EPPO, 1997), it was not logical to speculate the pine wood nematode in Wanding came from any Burmese source. Moreover, Wanding, the only site of infestation, lies in the west portion of the LRGR, which is regarded as natural barriers obstructing organisms from expanding by natural means (He et al., 2005). Therefore, it was also not logical to speculate that the nematodes came from adjacent areas in Yunnan. However, after a careful patrol of the initial infested pine stand, investigators discovered that the infested trees was centered an under-

constructing telecommunication facility, which consumed a considerable amount of electronics manufactured in eastern China (Z.Q. Li, pers. comm.). Hence, a reasonable hypothesis was conceived that the nematodes in Wanding were casually introduced with non-local *M. alternatus* populations hidden in the wood-packaging materials. Providing that *M. alternatus* from different localities possess different genetic profiles, the non-local individuals can then be distinguished by analyzing the genetic differences on population level, and the source can be traced by comparing the genetic profiles with the samples taken from the suspected region.

4. Validating the hypothesis – Theory and experiment

4.1 Molecular achievements on *Monochamus*

Although *Monochamus* species are important forest pests across the Eurasia, research employing molecular techniques were quite limited compared to other arthropod pests. Cesari et al. (2005) started the first molecular taxonomy research on seven *Monochamus* species by using the combined mtDNA data of *cox1* and *12s* genes. Afterward, such research were quickly developed in Japan, where *M. alternatus* had become a threat to forestry. Kawai et al. (2006) published a paper on the genetic structure of 27 populations of *M. alternatus* from Japan and China (with 25 populations from Japan and two populations from China). Shoda-Kagaya (2007) published paper on microsatellite markers. Then, Koutroumpa et al. (2009) conducted a criticizing research which revealed *Numts* resembling the *cox1* and *cox2* genes of *M. galloprovincialis* and *M. sutor*, alerting scientists that precautions are necessary when applying the gene markers from the mitogenome. There was no such research on *M. alternatus* in China published by the end of 2009.

4.2 Designing the experiment

Appropriate sampling strategy requires samples to be taken from populations scattered in the entire research range, and for each population, sufficient individuals should be collected to represent it (S.Y. Chen & Y.P. Zhang, 2006). Based on this, seven populations representing *M. alternatus* from central, southern, southwestern, and northern Yunnan were selected, in which three populations were chosen from the southwestern Yunnan where the pine wood nematodes were reported. In an attempt to determine the source of the pine wood nematode, two reference populations from outside Yunnan were set, with one from Hubei Province representing central China and the other from Zhejiang Province representing eastern China (Fu et al., 2010; Fig. 2). Adult *M. alternatus* were collected by flight traps baited with barkborer lure (Chinese Academy of Forestry, Zhejiang, China).

Choosing the correct genetic marker can minimize problems in data analysis (Simon et al., 1994) and maximize the future potential of cross references with data published by other researchers. Population phylogeny requires gene markers with moderate evolutionary rate, free of recombination, which means that mtDNA, a maternal heritage genome without recombination, is the ideal choice. Considering that there has been no report on *Numts* in *M. alternatus*, which can also be detected and excluded by strict data examination when encountered. The prefunding research by Kawai et al. (2006) resolved a clear population phylogeny using a gene fragment of *cox2*; the research discussed here chose the same *cox2* marker.

The same PCR primers developed by Roehrdanz (1993) and Kawai et al. (2006) were applied, and the PCR reaction was performed by the protocols described by Kawai et al. (2006). Bioinformatic and statistic software like DAMBE 5.0.7 (Xia & Xie, 2001), MEGA 4.0 (Tamura et al., 2007), SAMOVA 1.0 (Dupanloup et al., 2002), AMOVA 3.1 (Excoffier et al., 2005), and SSPS 13.0 (SPSS Inc., Illinois, US) were applied to analyze haplotype assemblage, Kimura two-parameter (K2P) distance (Kimura, 1980), NJ phylogenetic reconstruction (Saitou & Nei, 1987), population grouping, and multidimensional scaling (MDS) (Lessa, 1990). The mapping of haplotype distribution was performed in AcrView 3.3 (ESRI, USA).

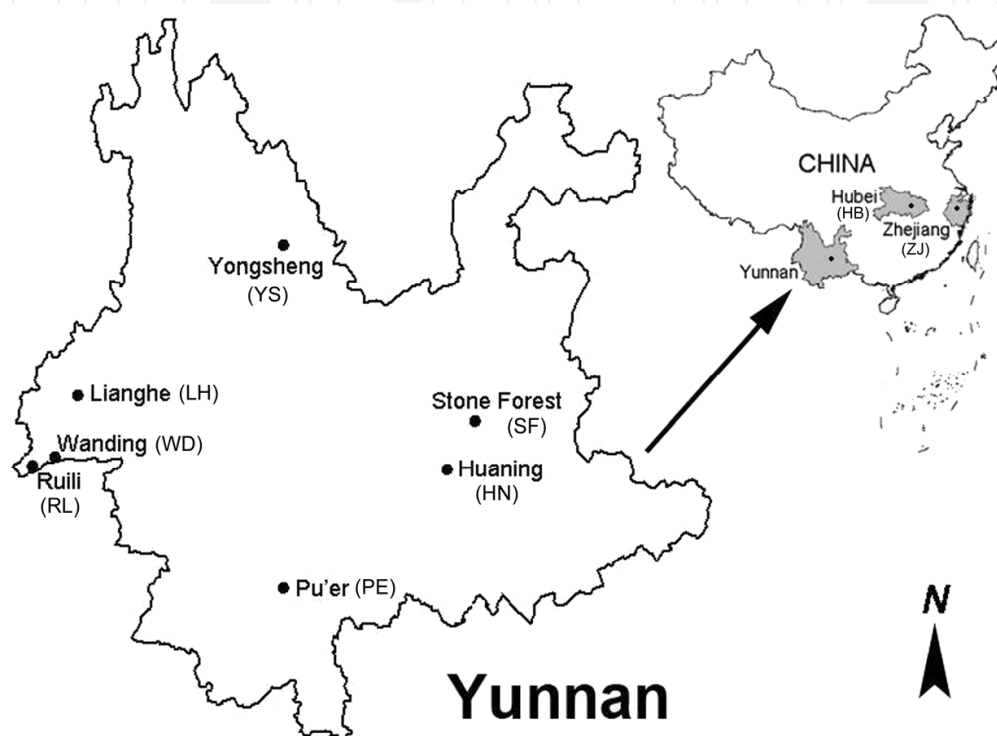


Fig. 2. The sampling sites of *M. alternatus* in Yunnan, Hubei, and Zhejiang. Dots on the map of China represent the capital cities of the three provinces. After Fu et al. (2010).

5. Validating the hypothesis – Molecular evidences

5.1 Haplotype assemblage

Eighteen haplotypes were defined by 22 polymorphic sites in the 565 bp *cox2* sequence, with haplotypes 1, 2, and 5 showing much higher frequencies. The distribution pattern of these 18 haplotypes was locality related: haplotypes 1 to 4 were widely distributed, dominating most of the central, northern, and southern Yunnan; haplotypes 6 to 18 were found within single localities. Haplotype 5 was shared by populations from southwestern Yunnan and Zhejiang; however, haplotypes 1 to 4 were neither shared by population from Hubei nor population from Zhejiang; and haplotypes 12 to 15 were only found in Hubei population. Further analysis of the haplotypes derived from the three populations in southwestern Yunnan discovered a high ratio of haplotype 5: with 69.2%, 70.0%, and 71.4% in Ruili, Wanding, and Lianghe populations, respectively. The matrix of shared haplotypes showed a boundary between the three populations from southwestern Yunnan and the remaining populations, with the shared haplotypes being confined within each population group and the unique

haplotypes being presented only in two of the populations from southwestern Yunnan (Table 1). When mapping haplotype frequencies into pie charts, a more obvious population boundary as well as the existence of non-local individuals in three populations from southwestern Yunnan can be observed (Fig. 3).

Population	PE	RL	WD	HN	SF	LH	YS	ZJ	HB
Pu'er									
Ruili	0								
Wanding	0	1							
Huaning	1	0	0						
Stone Forest	2	0	0	2					
Lianghe	2	1	1	1	2				
Yongsheng	1	0	0	1	1	1			
Zhejiang	0	1	1	0	0	1	0		
Hubei	0	0	0	0	0	0	0	0	
Unique haplotypes	0	3	3	0	0	0	0	3	4

Table 1. Matrix of shared haplotypes of the nine *M. alternatus* populations (below diagonal) and the numbers of unique haplotypes (last row). Population codes correspond to those in Fig. 2.

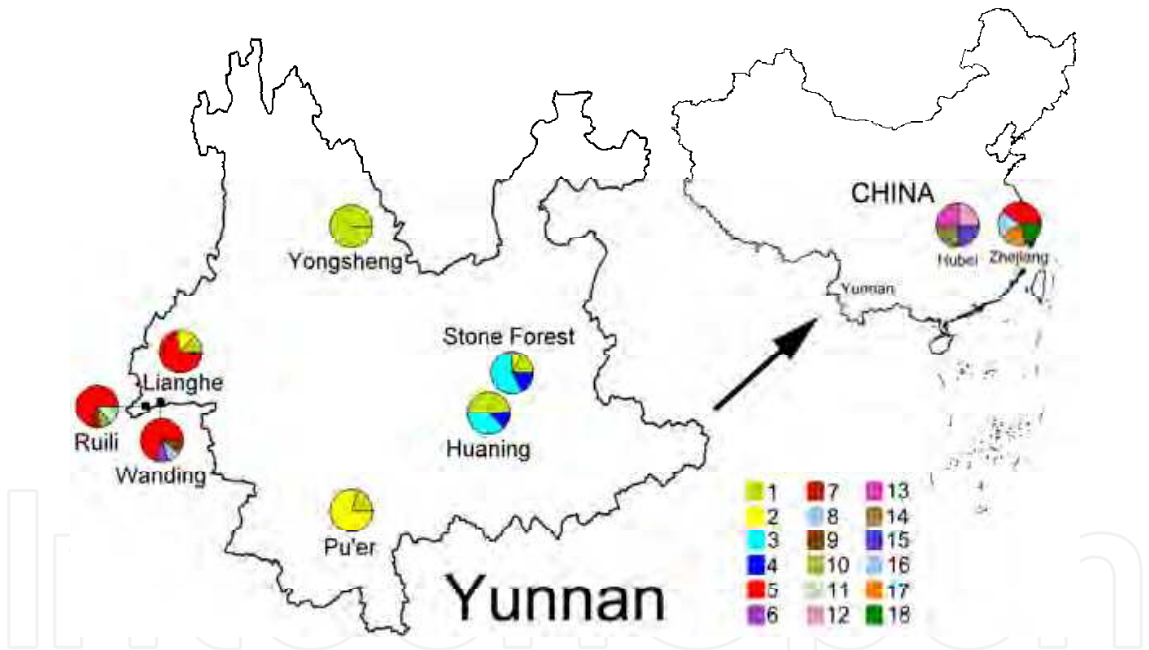


Fig. 3. Mapping of haplotype frequencies of all nine populations of *M. alternatus*.

5.2 Genetic distance

The Kimura two-parameter (K2P) distance varied from 0.0014 to 0.0132, with the distance between Pu'er (southern Yunnan) and Yongsheng (northern Yunnan) being the minimum and that between Stone Forest (central Yunnan) and Hubei being the maximum (Table 2). The K2P distances among populations from Pu'er, Huaning, Stone Forest, and Yongsheng and among populations from Ruili, Wanding, and Lianghe showed close genetic connection; but the K2P distances between any given population from Pu'er, Huanian, Stone Forest, or Yongsheng and that from Ruili, Wanding, or Lianghe suggested otherwise (Table 2). Hence,

the seven populations sampled from Yunnan could be divided into two groups, hereafter designated as the SPG, the southwestern population group containing populations from Ruili, Wanding, and Lianghe, and the RPG, the remaining population group containing populations from Pu’er, Huaning, Stone Forest, and Yongsheng. The grouping result was then supported by the multidimensional scaling (MDS) analysis (Fig. 4). Notably, both the K2P distances and the MDS analysis showed a close relationship between the population from Zhejiang and all populations from the SPG, however, the relationship between the population from Hubei and any population from both the SPG and the RPG was much greater (Table 2; Fig. 4).

Site	PE	RL	WD	HN	SF	LH	YS	ZJ	HB
PE		0.0037	0.0035	0.0012	0.0015	0.0028	0.0013	0.0037	0.0040
RL	0.0084		0.0007	0.0036	0.0040	0.0012	0.0034	0.0008	0.0024
WD	0.0084	0.0018		0.0035	0.0038	0.0012	0.0032	0.0009	0.0024
HN	0.0020	0.0090	0.0090		0.0013	0.0029	0.0012	0.0037	0.0040
SF	0.0022	0.0101	0.0101	0.0022		0.0032	0.0020	0.0040	0.0042
LH	0.0064	0.0027	0.0031	0.0071	0.0081		0.0026	0.0013	0.0025
YS	0.0014	0.0070	0.0070	0.0020	0.0031	0.0053		0.0034	0.0038
ZJ	0.0093	0.0021	0.0026	0.0098	0.0109	0.0035	0.0079		0.0027
HB	0.0122	0.0070	0.0077	0.0123	0.0132	0.0084	0.0107	0.0086	

Table 2. Matrix of the Kimura two-parameter (K2P) distance (below diagonal) and standard errors (above diagonal) of between populations of *M. alternatus*. Population codes correspond to those in Fig. 2. Data source: Fu et al. (2010).

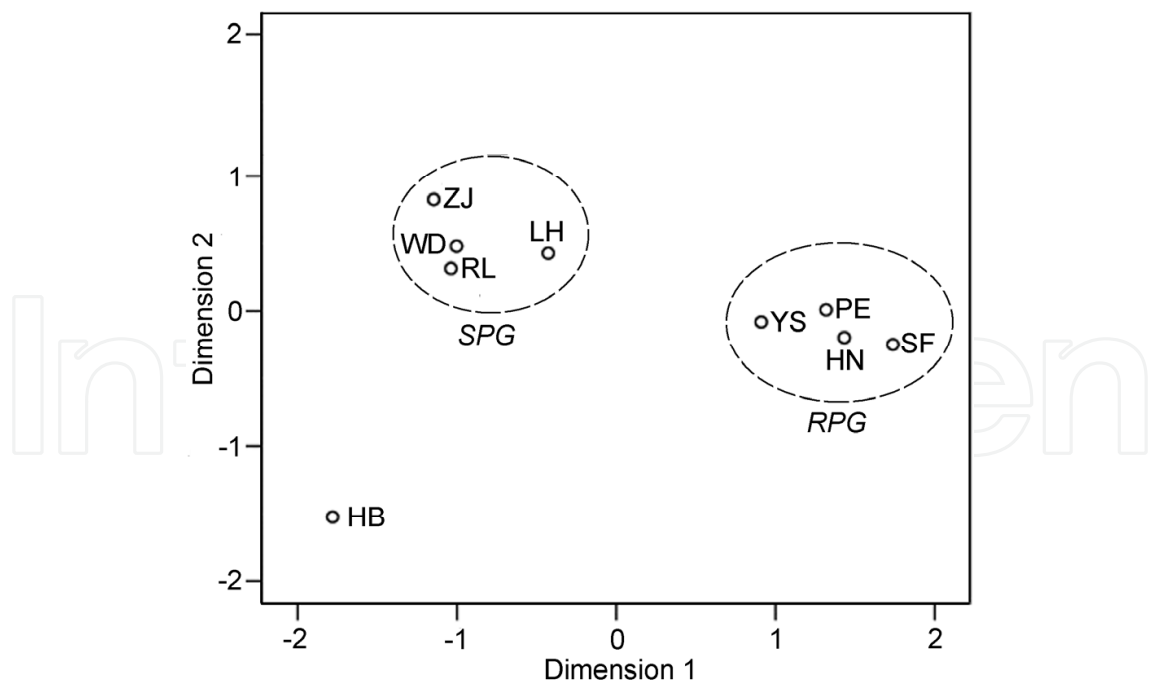


Fig. 4. The multidimensional scaling (MDS) plots of *M. alternatus* populations at different locations based on Kimura two-parameter (K2P) distances. SPG, the southwestern population group; RPG, the remaining population group. Population codes correspond to those in Fig. 2. After Fu et al. (2010).

5.3
Phylogenetic cladistics

The neighbor-joining (NJ) phylogenetic tree based on the K2P distance of the 18 haplotypes resolved three major clades designated from A to C (Fig. 5). Clade A contained six populations from Yunnan, with four from northern, central, and southern portion, and only one sample from Wanding, southwestern portion was included. Clade B contained the three populations from southwestern Yunnan and the population from Zhejiang, eastern China. However, no sample from other portions of Yunnan grouped in clade B. Clade C only contained the population from Hubei, central China. The topological structure of the NJ phylogenetic tree suggests that the seven populations consisted of two groups, the RPG and the SPG, regardless of the connection between them by a few samples from Wanding and Lianghe. It also suggested that the genetic profile of the population from Hubei is different from the remaining ones involved in the research.

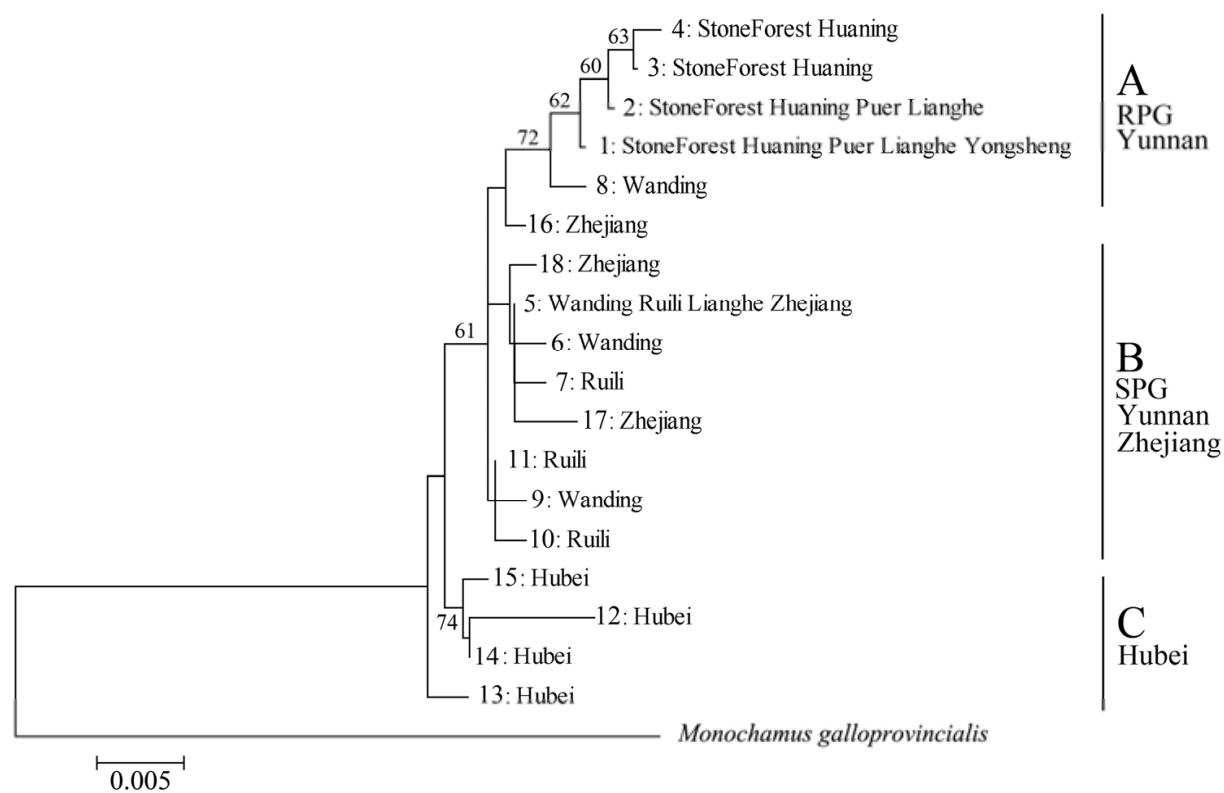


Fig. 5. The neighbor-joining (NJ) phylogenetic tree of the 18 haplotypes of *M. alternatus*, numbers above the branches represent the bootstrap values (> 50). After Fu et al. (2010).

5.4
Population genetic structure

SAMOVA and AMOVA analyses were applied to verify the genetic structure of the seven populations from Yunnan. When the number of groups (*K*) grows and the *F*_{CT} value reaches the plateau, the SAMOVA analysis yields the optimal result. In this research, the *F*_{CT} value reached the maximum (*F*_{CT} = 0.73) when *K* = 2, with populations from Pu'er, Huaning, Stone Forest, and Yongsheng being one group and populations from Ruili, Wanding, and Lianghe being the other (Fig. 6), supporting the grouping result mentioned previously. The grouping was then tested by the AMOVA analysis, which indicated that 72.54% of the variation

occurred between the two groups, 21.86% occurred within populations, and only 5.60% occurred among populations within groups (Table 3). The relatively high variation within populations was also mirrored by the haplotype assemblage that most of the populations possessed multiple haplotypes, especially the three populations from southwestern Yunnan rich in unique haplotypes (Table 1; Fig. 3).

Source of variation	d. f.	Variance components	Percentage of variation	F	P
Between groups	1	1.725	72.54	$F_{CT} = 0.73$	0.025
Among populations within groups	5	0.133	5.60	$F_{SC} = 0.21$	0.001
Within populations	54	0.520	21.86	$F_{ST} = 0.78$	0.000
Total	60	2.378	100	--	--

Table 3. AMOVA analysis results. Data source: Fu et al. (2010).

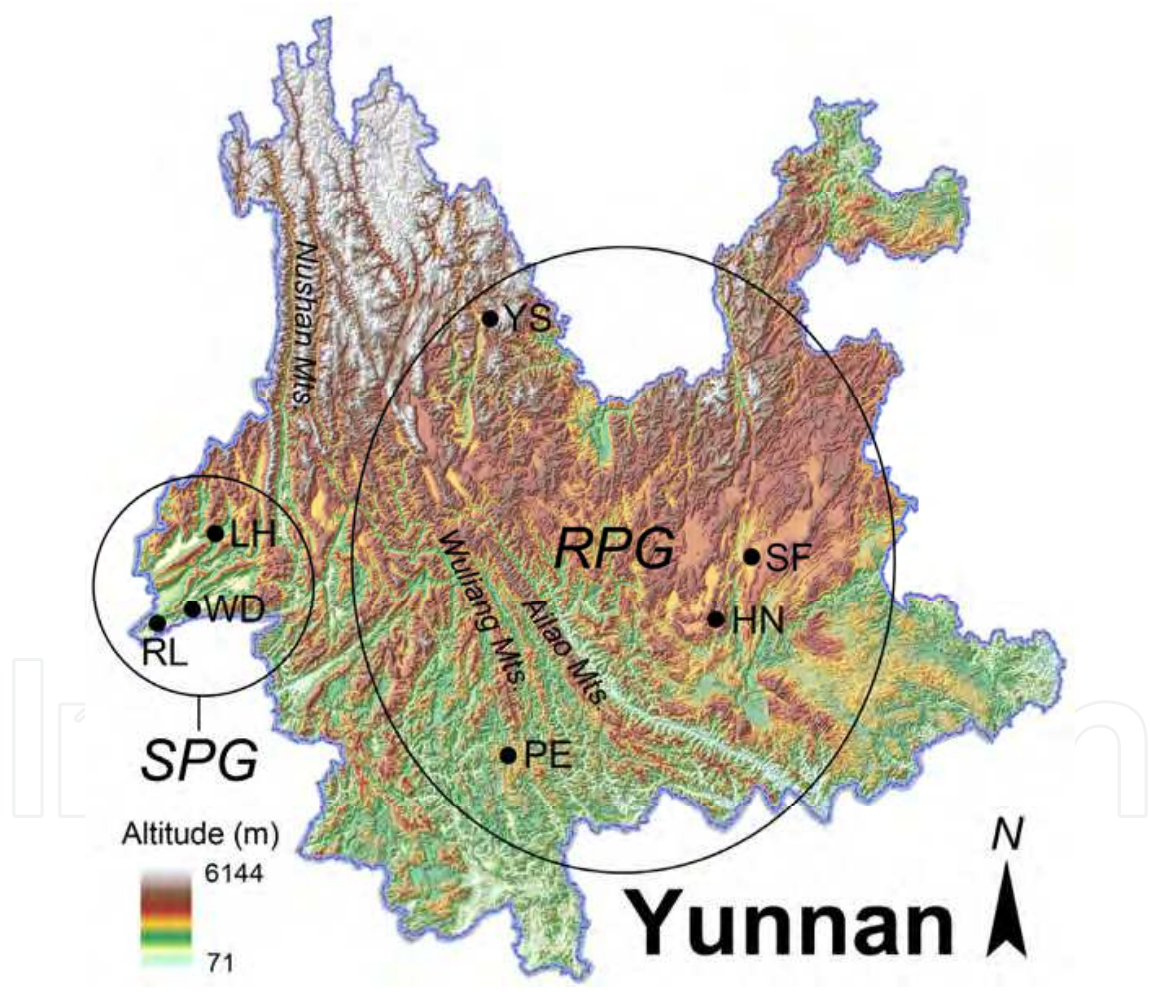


Fig. 6. The terrain of Yunnan and the grouping result of seven populations of *M. alternatus* determined by SAMOVA and AMOVA analyses with major geographical barriers marked (Ailao Mountains, Wuliang Mountains, and Nushan Mountains). SPG, the southwestern population group; RPG, the remaining population group. Population codes correspond to those in Fig. 2.

6. Determination of non-local *M. alternatus*

The experiment provided molecular evidence strongly supporting the following two hypotheses: 1) the genetic structure divides the seven *M. alternatus* populations from Yunnan into two groups, the southwestern population group (SPG) and the remaining population group (RPG), and 2) it is most likely that there were non-local individuals of *M. alternatus* in the three populations sampled from southwestern Yunnan, as these populations shared one of the dominant haplotypes of the reference population from Zhejiang. However, in an attempt to finally determine the identity of non-local *M. alternatus*, the relationship between such genetic divergence and the natural geographical characteristics of Yunnan should be considered thoroughly.

Genetic divergence has been frequently discussed in terms of natural geographical barriers (Yagi et al., 2001; Shoda-Kagaya, 2007; Shi et al., 2010). Yunnan is a typical LLPR with complex terrain, where numerous population phylogenetic studies on different insects have revealed the role that geographical barriers play in obstructing the gene flow among populations from various localities (Shi & Ye, 2004; Liu et al., 2007; P. Chen & Ye, 2008; Shi et al., 2010). Basically, the mountain ranges in the LRGR portion of Yunnan are considered effective geographical barriers, which restrict gene flow in the latitudinal direction.

Since the natural environment of Yunnan is able to cause genetic divergence, the separation of two population groups could be either the result of natural geographical barriers or the consequence of introduced non-local sources. The seven sampling sites in Yunnan were separated by great mountain ranges. Ruili, Wanding, and Lianghe were separated from other populations by Nushan Mountains, Pu'er was separated from Stone Forest, Huaning, and Yongsheng by Wuliang and Ailao Mountains (Fig. 6). But it is interesting to note that the genetic distances among populations from the RPG were limited to 0.0014 to 0.0031 (Table 2), the while genetic variation among them comprised only 5.6% of the total divergence (Table 3), and they shared all of the haplotypes defined by the samples taken from them (Table 1; Fig. 3). Hence, the geographical barriers such as Wuliang and Ailao Mountains had not caused sufficient genetic divergence among these populations. Judging from the phylogenetic tree (Fig. 5), these four populations were latest diverged, which may represent a short inhabitation history of *M. alternatus* in Yunnan under natural condition. To the contrast, the genetic distances between populations from the SPG and the RPG were much higher than that within each group, varied from 0.0064 to 0.0101 (Table 2), the genetic variation reached 72.54% (Table 3), and most of the unique haplotypes were defined (Table 1). Given the discussion above, the geographical barriers can not explain such divergence.

Assuming that the SPG and the RPG were two genetically independent sources, there must be gene flows among these populations in the course of evolution, which will be detected by shared haplotypes. It is noticeable that population from Lianghe shared two haplotypes with populations from the RPG; however, no haplotype from the SPG was shared by any other populations. Given that the individuals from the RPG were able to "migrate" into the SPG, the same phenomenon should have happened the other way around. Another assumption was that the genetic divergence between the SGP and the RPG was caused by introduction of non-local *M. alternatus*. The haplotype distribution demonstrated that about 90% individuals of the southwestern populations shared haplotype 5 with population from Zhejiang (Table 1; Fig. 3), and the phylogenetic tree indicated a close genetic connection

between haplotype 5 and those unique haplotypes (Fig. 5), which had been regarded as a symbol of establishment of non-local populations (Slatkin & Hudson, 1991; J. Hu et al., 2008).

As mentioned previously, the pine wilt disease in Wanding was reported four years after the arrival of electronics with wood packaging materials from eastern China where the pine wood disease was most severe. Considering the characteristics of the immature stages of *M. alternatus* and its association with *B. xylophilus*, it was possible that the un-treated, infested wood packaging materials disposed casually near the construction site became the source of infestation. This is not only would help to explain the significant genetic divergence between the SPG and the RPG in Yunnan, but also can explain the genetic connection between the SPG and the population from Zhejiang instead of Hubei.

7. Conclusion

The research proved the existence of non-local *M. alternatus* in southwestern Yunnan by utilizing the modern molecular techniques. It is the first, yet not only an isolated study on the population phylogeny of the *Monochamus* longhorn beetles in China, but also another exemplar of applying the molecular techniques to the research of biological invasion. Apart from demonstrating the feasibility of detecting non-local populations, it also provided an insight that, unlike the dispersal of other invasive alien species, such as fruit flies, the spread of pine wood nematodes in mainland China was mostly due to human-aided relocation of infested, un-treated wood packaging materials. Therefore, preventing the infested wood materials from circulating or being transported may become the most important checkpoint of preventing the pine wilt disease from expanding.

8. Future research and objectives

In the research discussed here, the modern molecular techniques provided strong evidence which proved the dispersal of the pine wood nematodes in southwestern Yunnan was caused by the casual introduction of non-local Japanese pine sawyer, *Monochamus alternatus*, and even helped to identify the possible origin of those non-local *M. alternatus* by combining both of the molecular data and the historical facts of telecommunication construction in the infested area. The most advanced technology nowadays allows the scientists to identify the pine wood nematodes directly from either the wood sample or the beetles (X.R. Wang et al., 2010; X.R. Wang et al., 2010; Y.Q. Hu et al., 2011). By utilizing such innovative methods, the foresters and plant quarantine staff are able to turn the identification of invasive organisms from sheer speculation to solid evidence. By promoting such concept along with the modern molecular techniques, not only will future law enforcement benefit, but also the regional and international trading embargos surrounding quarantine and/or invasive organisms shall be solved in a proper manner.

Apart from identifying non-local populations, population phylogeny can also be used to study the historical dispersal (including both natural dispersal and human-aided relocation) of insects, as suggested by research mentioned earlier (Kawai et al., 2006; Shoda-Kagaya, 2007; Shi et al., 2010). Since China has a 28-year dispersal history of the pine wilt disease, the dispersal pattern of the pine wood nematode as well as its key vector, *M. alternatus*, should be an interesting field to research. However, to date, there is only one paper on the dispersal pattern of the nematodes in China (Sun et al., 2008). Currently, the senior author and his

colleagues are conducting a panoramic research on the population phylogeny of *M. alternatus* sampled from multiple localities in mainland China. The result of this research is to partially answer the long-asked question regarding the dispersal pattern of *M. alternatus* during the spread of the pine wilt disease in China. And as it is now possible to test the genetic profiles of *B. xylophilus* and *M. alternatus* together, future studies on the dispersal pattern and original sources of the pine wood nematodes in China can be revealed more thoroughly.

9. Acknowledgements

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