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ORIGINAL ARTICLE

Pine wilt disease in Yunnan, China: Evidence of non-local pine sawyer *Monochamus alternatus* (Coleoptera: Cerambycidae) populations revealed by mitochondrial DNA

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Abstract *Monochamus alternatus* (Hope) specimens were collected from nine geographical populations in China, where the pinewood nematode *Bursaphelenchus xylophilus* (Steiner et Buhrer) was present. There were seven populations in southwestern China in Yunnan Province (Ruili, Wanding, Lianghe, Pu'er, Huaning, Stone Forest and Yongsheng), one in central China in Hubei Province (Wuhan), and one in eastern China in Zhejiang Province (Hangzhou). Twenty-two polymorphic sites were recognized and 18 haplotypes were established by analyzing a 565 bp gene fragment of mitochondrial cytochrome oxidase subunit II (CO II). Kimura two-parameter distances demonstrated that *M. alternatus* populations in Ruili, Wanding and Lianghe (in southwestern Yunnan) differed from the other four Yunnan populations but were similar to the Zhejiang population. No close relationship was found between the *M. alternatus* populations in Yunnan and Hubei. Phylogenetic reconstruction established a neighbor-joining (NJ) tree, which divided haplotypes of southwestern Yunnan and the rest of Yunnan into different clades with considerable bootstrapping values. Analysis of molecular variance and spatial analysis of molecular variance also suggested significant genetic differentiation between *M. alternatus* populations in southwestern Yunnan and the rest of Yunnan. Our research suggests that non-local populations of *M. alternates*, possibly from eastern China, have become established in southwestern Yunnan.

Key words mitochondrial DNA, non-local vector, pine wilt disease

Introduction

Monochamus alternatus (Hope) (Coleoptera: Cerambycidae: Lamiinae) occurs widely in China, Korea, Japan, Laos and Vietnam. It is the key vector of the exotic pinewood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer), throughout Asia (Mamiya & Enda, 1972; Kobayashi *et al.*, 1984; Ning *et al.*, 2004). This nematode is the causal agent of pine wilt disease, which has caused widespread pine (*Pinus* spp.) mortality in Asia and severely impacted Japanese forestry (Shibata, 1999). In China, the pinewood nematode was first recorded in Nanjing in 1982 (Ning *et al*., 2004). Since then and up to 2007, it has been found in 113 cities/counties in 12 provinces, and has resulted in tremendous timber losses (Ning *et al*., 2004; SFA, 2007).

Pinewood nematodes are able to locate pupae of *M. alternatus* in infested pine trees and enter the tracheae of the newly eclosed adult beetles prior to their emergence from the host tree. Then, the nematodes are carried to a new host tree within the beetles' tracheae. During the adult beetles' feeding and oviposition on new host trees, the nematodes drop from the tracheae, enter the feeding

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wounds, and thereby initiate infection in a new host. In this way, nematodes spread throughout a pine forest. However, for long-distance dispersal, human activity such as transportation of infested logs, lumber or wood packaging can serve as an important pathway for the movement of wood-infesting tree pests (Haack, 2006; Sun *et al*., 2008; Haack *et al*., 2010).

The life stages of *Monochamus* species are commonly transported in wood packaging materials. For example, from 1985 to 2000, *Monochamus* was the most commonly intercepted genus of Cerambycidae in wood packaging from China at ports in the United States (Haack, 2006). Given that *Monochamus* individuals have been commonly moved in Chinese wood packaging in international trade, it is logical to assume that they have also been moved in wood packaging within China.

In 2004, pinewood nematode-infested *Pinus kesiya* var. *langbianensis* (A. Chevalier) trees were recorded in Wanding, Ruili Municipality in southwestern Yunnan Province, which was considered to be the first occurrence of this nematode in Yunnan (SFA, 2007). Considering that pinewood nematodes had not previously been found in Yunnan, the infested wood-packaging materials from outbreak areas in eastern China were suspected as a possible source. During 2000–2007, the eastern portion of China was the principal outbreak area for pinewood nematode and Zhejiang Province was one of the major outbreak areas (Ning *et al*., 2004; SFA, 2004, 2005, 2006, 2007). Since 2001, large amounts of wood-packaging have been transported from Zhejiang Province to Wanding (Yunnan Province) in association with telecommunication construction projects. Soon after that, many pine forests near to those projects were found to be infected with pine wilt disease (Shao-Ji Hu, pers. comm., 2007). If this situation were true, it is possible that there would be non-local *M. alternatus* populations in Yunnan Province, and the genetic structure of the non-local *M. alternatus* population would be different from local Yunnan *M. alternatus* populations as detected by phylogenetic comparisons.

Molecular techniques using genetic data are effective in intra-specific and phylogenetic research (Boge *et al*., 1994; Hidayat *et al*., 1996; Brown *et al*., 1997; Miller *et al*., 1999; Kethidi *et al*., 2003). The COII gene is a coding sequence for cytochrome oxidase subunit II located in mitochondrial DNA. Insect COII genes are limited to a length of 670–690 bp with a medium evolutionary rate. It is commonly applied to inter- or intra-specific phylogenetic studies (Emerson & Wallis, 1995; Gómez-Zurita *et al*., 2000; Wang & Yang, 2002; Kawai *et al*., 2006). In this study, we utilized partial COII gene to reveal phylogenetic differences among *M. alternatus* populations in Yunnan Province and to evaluate the possibility that the pinewood nematodes in Yunnan arrived as a result of the introduction of non-local populations of *M. alternatus*.

Materials and methods

Sample collection and preparation

We selected seven sample sites in Yunnan Province, China (Table 1; Fig. 1) to collect adult specimens of *Monochamus alternatus* during 2005–2007. All sample sites were forest stands where local pine trees were *Pinus yunnanensis* Franchet in northwestern and central Yunnan and *P. kesiya* var. *langbianensis* in southern and southwestern Yunnan. In an attempt to detect non-local *M. alternatus* populations, we also sampled specimens from two severe outbreak areas of pinewood nematode,

Table 1 Summary information of sampling sites in Yunnan Province, China.

Site	Code	Latitude	Longitude	Alt. (m)	Host†	Climate‡	Preservation§	No. adults
Pu'er	PE	22°45′N	100°00′E	1 396	PK	s. subtropics	AR ethanol	5
Ruili	RL	24°01′N	97°50′E	806	PK	n. tropics	AR ethanol	13
Wanding	WD	24°05′N	98°04′E	945	PK	n. tropics	Dehydration	10
Huaning	HN	24°11′N	102°55′E	1 647	PY	n. subtropics	Dehydration	8
Stone Forest	SF	24°45′N	103°15′E	1 694	PY	n. subtropics	Dehydration	12
Lianghe	LH	24°48′N	98°17′E	1 119	PK	s. subtropics	Dehydration	7
Yongsheng	YS	26°41′N	100°43′E	2 264	PY	s. temperate	AR ethanol	6

Geographical data were recorded by GPS device (Garmin eTrex Vista, software version 3.20, Olathe, KS, US).

†Host plants: PK, *Pinus kesiya* var. *langbianensis*; PY, *Pinus yunnanensis*.

‡Climate: s. subtropics, southern subtropics; n. tropics, northern tropics; n. subtropics, northern subtropics; s. temperate, southern temperate.

§Preservation: AR ethanol, analytical reagent ethanol.

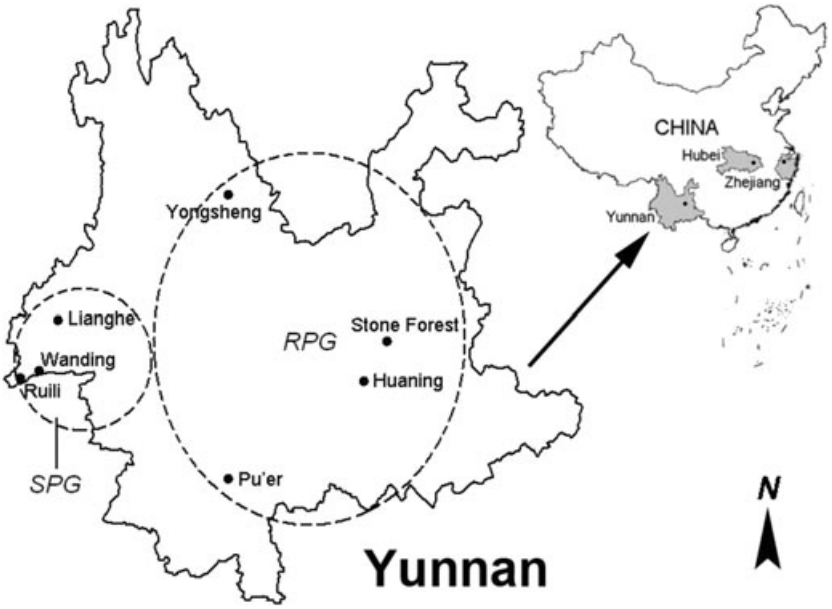

Fig. 1 Sampling sites in Yunnan Province. Dots on the map of China represent the capital cities of the three corresponding provinces. SPG, the south-western population group; RPG, the remaining population group. [Yunnan S(2009)043].

Hubei Province (Code: HB) in central China and Zhejiang Province (Code: ZJ) in eastern China, in 2007.

Adult *M. alternatus* specimens were captured in beetle traps [Chinese Academy of Forestry (CAF), Zhejiang, China; Fujian Academy of Forestry Sciences (FAFS), Fujian, China] with Barkborer Bait (CAF) or FJ-Ma-02 Bait (FAFS), and trap logs made from recently cut, local pine trees. The principal attractant in the lure was α-pinene. Beetle traps were placed in pine forests from late March to early October each year, and were checked weekly for specimens. At the time of collection, some beetles were alive while others had already died. Collected *M. alternatus* specimens were dehydrated or preserved in analytical reagent ethanol (AR ethanol) at room temperature. Details on the Yunnan sample sites and number of *M. alternatus* collected per sample site are shown in Table 1. In an attempt to obtain high-quality mitochondrial DNA, each beetle was dissected to obtain muscle tissue from its mesothorax. The muscle tissue was ground in a 1.5-mL centrifuge tube at room temperature.

DNA protocols

Genomic DNA was extracted with a tissue/cell genomic DNA isolation kit (Watson Biotechnologies Inc., Shanghai, China). Products were preserved at −20°C.

The COII gene sequence was prepared by polymerase chain reaction (PCR) on a Biometra T-Gradient PCR device (Whatman, Dassel, Germany). An approximate 600 bp fragment of mtDNA, which includes the COII sequence, was amplified. The PCR program consisted of an initial denaturation at 93°C for 3 min; followed by 30 cycles of denaturation at 93°C for 30 s, annealing at 47°C for 60 s, and elongation at 72°C for 2 min; then a final elongation at 72°C for 2 min. The PCR reaction was applied in a 22.75 μL system which contained 2.6 μL of 10 × PCR buffer (Shanghai Promega, Shanghai, China), 2.6 μL of $MgCl_2$ (25 mmol/L; Shanghai Promega), 4.1 μL of dNTP mixture (2.5 mmol/L each; Fermentas, EU), 0.5 μL of *Taq* DNA polymerase (5 U/μL; Shanghai Promega), and 0.25 μL of each of forward and reverse primers (20 μmol/L; SBS Genetech Co., Ltd., Beijing, China). The applied primers were COII-Croz (sequence: 5′-CCA CAA ATT TCT GAA CAT TGA CC-3′) (Roehrdanz, 1993) and $tRNA^{Leu-F}$ (sequence: 5′-GTG CAA TGG ATT TAA ACC CC-3′) (Kawai *et al*., 2006).

PCR products were purified and sequenced by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd (Shanghai, China). Sequencing reactions were carried out in both directions on an ABI Prism 3730xl automatic sequencer (Applied Biosystems, Foster City, CA, USA).

Data analysis

Sequences were aligned using CLUSTAL W (Thompson *et al*., 1994) in BioEdit 7.0.9 (Hall, 1999). Unique

Table 2 Nucleotide variations of 22 polymorphic sites of 18 haplotypes (HT).

HT	Polymorphic site																					
	7	22	64	67	70	160	181	182	262	268	319	328	343	436	442	448	461	481	526	556	559	565
A	T	T	C	T	T	T	A	G	C	C	C	T	C	T	C	A	T	T	A	C	T	T
B	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C
C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C
D	–	–	T	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C
E	–	–	–	–	–	–	–	–	T	–	T	–	–	–	T	–	–	–	–	T	–	–
F	C	–	–	–	–	–	–	–	T	–	T	–	–	–	T	–	–	–	–	T	–	–
G	–	–	–	–	–	–	–	–	T	–	T	–	–	–	T	–	–	–	T	T	–	–
H	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	–	–	–	–	T	–	–
I	–	A	–	–	–	–	–	–	T	–	–	–	–	–	T	–	–	–	–	T	–	–
J	–	–	–	C	–	–	–	–	T	–	–	–	–	–	T	–	–	–	–	T	–	–
K	–	–	–	–	–	–	–	–	T	–	–	–	–	–	T	–	–	–	–	T	–	–
L	–	–	–	–	–	–	–	A	T	T	–	C	T	C	T	–	–	A	–	T	–	–
M	–	–	–	–	–	–	G	–	T	–	–	–	T	–	T	C	–	–	–	T	–	–
N	–	–	–	–	–	–	–	–	T	T	–	–	T	–	T	–	–	–	–	T	–	–
O	–	–	T	–	–	–	–	–	T	T	–	–	T	–	T	–	–	–	–	T	–	–
P	–	–	–	–	–	–	–	–	T	–	T	–	–	–	–	–	–	–	–	T	–	–
Q	–	–	–	–	–	–	–	–	T	–	T	–	–	–	T	–	C	–	–	T	C	–
R	–	–	–	–	C	–	–	–	T	–	T	–	–	–	T	–	–	–	–	T	–	–

haplotypes were distinguished using DAMBE 5.0.7 (Xia, 2000; Xia & Xie, 2001). Pair-wise distances and standard errors between populations of each sampling site were calculated with Kimura two-parameter model (Kimura, 1980) in MEGA 4.0 (Tamura *et al*., 2007) and bootstrapped with 1 000 iterations. Multidimensional scaling (MDS) (Lessa, 1990) based on Kimura two-parameter distance was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, US). A phylogenetic tree was reconstructed by the neighbor-joining (NJ) (Saitou & Nei, 1987) method in MEGA 4.0, and bootstrap was performed to test the reconstruction with 1 000 iterations. The sequence of the same gene fragment form *Monochamus galloprovincialis* was queried from GenBank and used as an outgroup (accession number: EU599210). SAMOVA 1.0 (Dupanloup *et al*., 2002) was used to explore the genetic relationship among populations. Analyses of molecular variance (AMOVA) were processed in Arlequin 3.1 (Excoffier *et al*., 2005).

Results

Mitochondrial DNA

Sixty-one *M. alternatus* individuals were collected and analyzed from the seven Yunnan populations, with five individuals being the minimum sample size in Pu'er and 13 being the maximum sample size in Ruili. Seventy sequences containing the COII gene were aligned over 565 bp. Twenty-two sites were polymorphic (Table 2), comprising 3.9% of the total nucleotides. No deletion or insertion was detected. Nucleotide frequencies showed that overall A-T comprised 74.3% of the total nucleotide composition on average.

Haplotype distribution

Eighteen haplotypes were defined and designated from A to R based on polymorphic sites (Table 2), among which some were dominant and others were more local and specific: haplotypes A to D were widely distributed, dominating most of the central, western and southern portions of Yunnan Province; haplotypes F to R only existed within single localities (Table 3). Haplotypes A, B and E, which had much higher frequencies among all samples, were deposited in GenBank (accession numbers: EU274291, EU274292 and EU274293).

Kimura two-parameter distance and phylogenetic tree

Kimura two-parameter distances between populations of each sampling site varied from 0.001 4 to 0.013 2

Table 3 Haplotype (HT) distribution of all sampled *M. alternatus*.

HT	Sampling site									Total
	PE	RL	WD	HN	SF	LH	YS	ZJ	HB	
A	1	–	–	4	2	1	6	–	–	14
B	–	–	–	3	7	–	–	–	–	10
C	4	–	–	–	1	1	–	–	–	6
D	–	–	–	1	2	–	–	–	–	3
E	–	9	7	–	–	5	–	2	–	23
F	–	–	1	–	–	–	–	–	–	1
G	–	1	–	–	–	–	–	–	–	1
H	–	–	1	–	–	–	–	–	–	1
I	–	–	1	–	–	–	–	–	–	1
J	–	1	–	–	–	–	–	–	–	1
K	–	2	–	–	–		–	–	–	2
L	–	–	–	–	–	–	–	–	1	1
M	–	–	–	–	–	–	–	–	1	1
N	–	–	–	–	–	–	–	–	1	1
O	–	–	–	–	–	–	–	–	1	1
P	–	–	–	–	–	–	–	1	–	1
Q	–	–	–	–	–	–	–	1	–	1
R	–	–	–	–	–	–	–	1	–	1
Total	5	13	10	8	12	7	6	5	4	70

PE, Pu'er; RL, Ruili; WD, Wanding; HN, Huaning; SF, Stone Forest; LH, Lianghe; YS, Yongsheng.

(Table 4). Populations in Ruili, Wanding, and Lianghe (in south-western Yunnan) differed from the other populations in Yunnan Province (0.006 4 to 0.010 1); but showed a close relationship with the population from Zhejiang Province (0.002 1 to 0.003 5). The population from Hubei Province differed significantly from all of the other populations sampled (0.007 0 to 0.013 2). Multidimensional scaling (MDS) categorized populations into three defined clusters (stress = 0.029, RSQ = 0.99). Populations in Ruili, Wanding, Lianghe and Zhejiang showed similar genetic composition, populations in Pu'er, Huaning, Stone Forest and Yongsheng were in one cluster, and the population in Hubei differed from the other two clusters (Fig. 2).

The phylogenetic tree showed that halplotypes A, B, C, D and H (clade *a*), derived from specimens collected from

Table 4 Kimura two-parameter distances (lower left data) and standard errors (upper right data) between *M. alternatus* populations for all sampling sites.

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	

PE, Pu'er; RL, Ruili; WD, Wanding; HN, Huaning; SF, Stone Forest; LH, Lianghe; YS, Yongsheng.

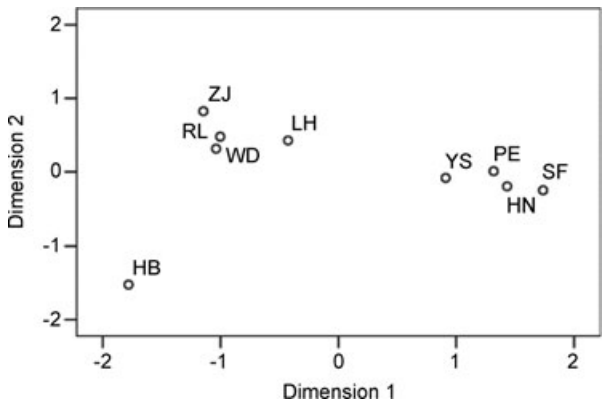

Fig. 2 Multidimensional scaling (MDS) plots of *M. alternatus* populations at different locations based on Kimura two-parameter distances. Location codes correspond to those in Table 1.

central (Huaning and Stone Forest), southern (Pu'er), and north-western Yunnan (Yongsheng), and a few specimens collected from south-western Yunnan, were divided from haplotypes E to G, I to K and P to R, all of which were derived from specimens collected from south-western Yunnan, as well as the individuals from Zhejiang. Clade *b* included haplotypes L and N, which were derived from specimens collected in Hubei (Fig. 3).

SAMOVA and AMOVA

SAMOVA divided the seven populations in Yunnan into two groups with the highest F_{CT} value ($F_{CT} = 0.725\,37$, $P = 0.029\,33$). One group (the south-western population group) was composed of populations from Ruili, Wanding and Lianghe, and the other population group was composed of populations from Pu'er, Huaning, Stone Forest and Yongsheng. AMOVA detected divergences between these two groups, among populations within each group, and within populations as well. The results showed that the variant percentage between the two groups comprised 72.5% of the total ($\Phi = 0.73$, $P = 0.025$). The within-population variant percentage was 21.9% ($\Phi = 0.78$, $P = 0.000$), while the among-population within groups variant percentage was only 5.6% ($\Phi = 0.21$, $P = 0.001$) (Table 5).

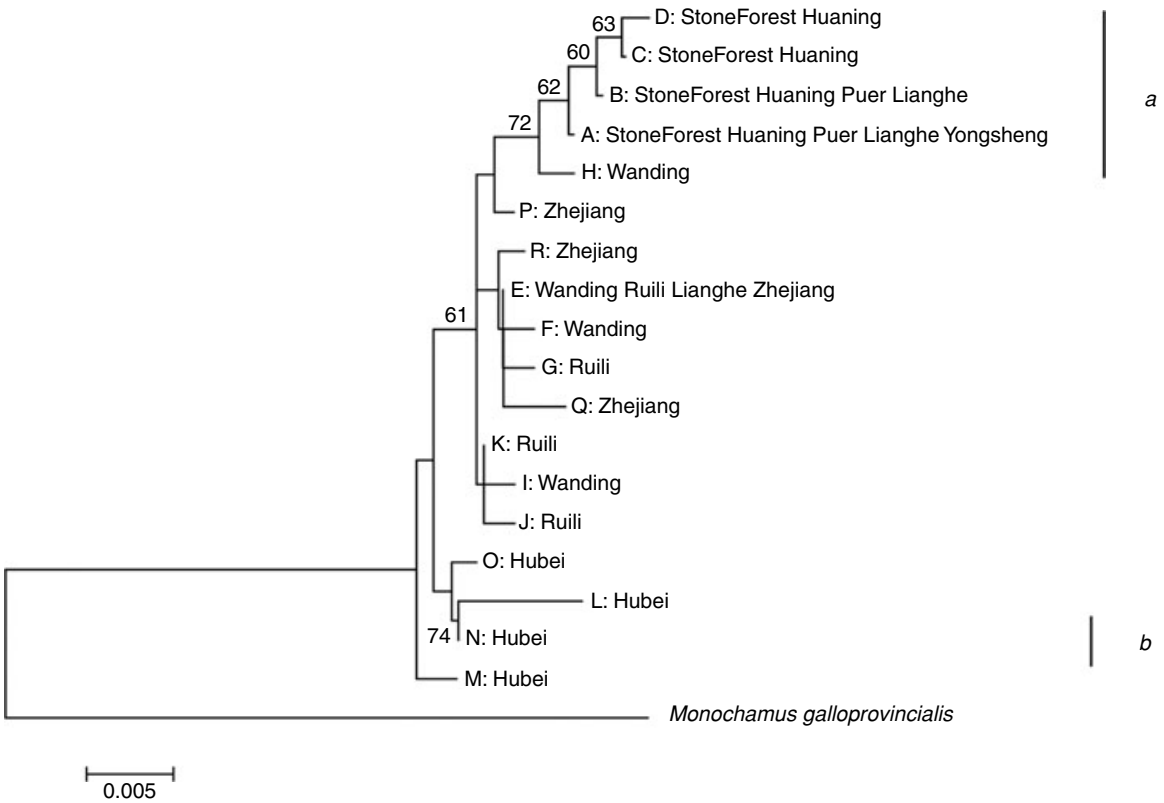

Fig. 3 Neighbor-joining (NJ) phylogenetic tree of 18 haplotypes of *M. alternatus* in Yunnan, China. Only bootstrap values above 50 are shown.

Table 5 Analysis of molecular variance results for *M. alternatus* populations sampled in Yunnan Province, China.

Source of variation	df	Variance components	Percentage of variation	Φ	P
Between groups	1	1.725 39	72.54	$\Phi_{CT} = 0.73$	0.025
Among populations within groups	5	0.133 16	5.60	$\Phi_{SC} = 0.21$	0.001
Within populations	54	0.520 07	21.86	$\Phi_{ST} = 0.78$	0.000
Total	60	2.378 62	100	–	–

Discussion

The pine sawyer populations in Yunnan Province were separated into two groups based on the genetic distances between populations (Table 4, Fig. 2): one was the south-western group that included populations from Ruili, Wanding and Lianghe; the other included populations from elsewhere in Yunnan (Pu'er, Huaning, Stone Forest and Yongsheng; Fig. 1). It was obvious that the genetic difference between the two groups was significantly higher than within the populations (Table 5); whereas the Kimura two-parameter distance data among populations was less within groups than between the two groups (Table 4).

Genetic divergence for most insect populations has usually been discussed in terms of geographical barriers (Hamelin *et al*., 2000; Yagi *et al*., 2001; Shoda-Kagaya, 2007; Schmidt *et al*., 2008). Yunnan Province is a mountainous area (94% mountains and hills), with five climatic types within its territory of 394 000 km^2. Elevation extremes vary from 76 m in south-eastern Yunnan to 6 740 m on mountain peaks in north-western Yunnan. More than five longitudinal mountain ranges run parallel, from west to east, in Yunnan. Shi and Ye (2004) demonstrated that the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), had genetically different geographical populations in Yunnan, which was likely due to Yunnan's mountain ranges reducing gene flow. However, the mountainous geography of Yunnan does not help explain the variation that we noted among the seven sample sites in Yunnan in the present study. For example, there are the Wuliangshan Mountains and Ailaoshan Mountains between southern (Pu'er) and central (Huaning and Stone Forest) Yunnan, the Diancangshan Mountains between central (Huaning and Stone Forest) and north-western (Yongsheng) Yunnan, and the Nushan Mountains between south-western (Ruili, Wanding and Lianghe) and other portions of Yunnan. The genetic distance among the populations of Pu'er, Huaning, Stone Forest and Yongsheng ranged from 0.001 4 to 0.003 1, comprising only 5.6% of total divergence among all the populations ($\Phi = 0.21$, $P = 0.001$). Therefore, geographical factors in Yunnan are not sufficient to explain the genetic divergence that we found between the two population groups. One possibility to explain this pattern is that the natural dispersal history of *M. alternatus* in Yunnan has not been sufficiently long to generate genetic divergence.

Alternatively, as supported by the haplotype distribution patterns, it appears that some individuals in the south-western Yunnan populations originated from non-local sources (Table 3). One possibility to explain this phenomenon is that all or most of the original founder individuals in south-western Yunnan were genetically independent from other parts of Yunnan in their history, and those rare individuals with shared haplotypes were immigrants from the remaining areas of Yunnan. If immigration could happen, then so could emigration from south-western Yunnan. However, the specific haplotypes of the south-western Yunnan populations have not been found elsewhere in Yunnan.

Another possibility is that originally there was only one homogenous population in Yunnan, but divergence between the two groups has occurred more recently after arrival of non-local individuals from outside Yunnan. Arrival of non-local individuals could later expand from a small founder population and result in the spread of unique haplotypes (Slatkin & Hudson, 1991; Hu *et al.*, 2008). Note that 90% of the southwestern samples shared haplotype E. Given the above discussion, it appears that the second possibility is more reasonable.

Moreover, as mentioned previously, the pinewood nematode was reported in Wanding 4 years after the initial arrival of large amounts of wood packaging materials from outside Yunnan (SFA, 2007). Comparing populations of Yunnan, Zhejiang and Hubei Provinces, we found that the genetic distances between south-western Yunnan and Zhejiang Province (0.002 1 to 0.003 5) were significantly closer than those between south-western Yunnan and Hubei Province (0.007 0 to 0.008 4). The geographical distance between Yunnan and Zhejiang is $\approx$ 1 800 km, compared with nearly 1 300 km between Yunnan and Hubei (distances were calculated as a straight line between the provincial capital cities) (Fig. 1). In fact, much wood packaging has entered Wanding during the past decade

from Zhejiang for large-scale telecommunications construction projects, while little has been imported from Hubei (Shao-ji Hu, pers. comm., 2007). If wood packaging had arrived in Wanding that was infested with both non-local populations of *M. alternatus* and pinewood nematodes, this could help explain why the molecular aspects of *M. alternatus* in south-western Yunnan appeared to be a mixture of both local and non-local individuals. In addition, the above scenario could explain the presence of pinewood nematode in south-western Yunnan.

Our study strongly suggests that *M. alternatus* individuals from eastern China arrived in south-western Yunnan and served as the original vectors of pinewood nematode. In order to more clearly identify the source of the non-local *M. alternatus* individuals and pinewood nematodes, more sampling sites in eastern China and possibly other parts of Asia should be collected and analyzed.

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