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Multiple Gene Genealogical Analyses of a Nematophagous Fungus *Paecilomyces lilacinus* from China[§]

Juan Li[†], Heng Li[†], Xiaoxu Bi, and Ke-Qin Zhang[*]

Laboratory for Conservation and Utilization of Bio-resources, and Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University, Kunming, Yunnan 650091, P. R. China

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***Paecilomyces lilacinus* is a geographically widespread nematophagous fungus and a promising biological control agent against plant parasitic nematodes. However, relatively little is known about its patterns of genetic variation through its broad geographic and ecological contexts. In this study, we analyzed the genetic variation of 2 virulence-associated genes (*PLS* and *PLC*) and 4 housekeeping gene fragments (*ITS*, *RPB1*, *RPB2*, and *β-tubulin*) among 80 *P. lilacinus* specimens collected from 7 locations in China. Various degrees of polymorphism and haplotype diversity were observed among the six gene fragments. However, no genetic differentiation was observed among the geographic populations, consistent with extensive gene flow among these geographic populations of *P. lilacinus* in China. Our analysis also suggested that clonal reproduction was the predominant mode of reproduction in natural populations of *P. lilacinus*.**

***Keywords*:** genetic diversity, biological control, haplotype, clonal reproduction

Introduction

Paecilomyces lilacinus is a common soil ascomycete fungus with nematophagous properties that has been examined over the past two decades for potential use as an agent for the control of root-knot nematodes and other plant parasitic nematodes in agricultural fields (Hewlett *et al*., 1988; Zaki and Bhatti, 1990; Kiewnick and Sikora, 2006). The application of *P. lilacinus* in such fields has increased the yield of many crop plants and lowered the population of nematode juveniles. Due to the significant promises of *P. lilacinus* in agricultural applications, many companies have been established to conduct applied research on this fungus. However, although some genetic variation studies (e.g. microsattelite and AP-PCR) have been conducted on other *Paecilomyces* species previously (Tigano-Milani *et al*., 1995a, 1995b; Chew *et al*., 1998; Fargues *et al*., 2002), none has conducted genetic variation study on *P. lilacinus*. The lack of knowledge about the population biology and genetics of this species in nature limited the effective application of *P. lilacinus* as biological control agents. A thorough understanding of the patterns of genetic variation and the evolutionary potentials in populations of *P. lilacinus* could speed up such applications.

Paecilomyces lilacinus destructs the eggshell of cysts and eggs of nematode by secreting many extracellular enzymes. For example, the subtilisin-like protease PLS has been purified from *P. lilacinus* and identified as a key virulence protease during the infection process of nematode eggs (Bonants *et al*., 1995). The encoding gene of PLS (GenBank number: L29262) has also been partially cloned from *P. lilacinus* by Bonants *et al.* (1995) and its corresponding whole gene sequence has been obtained through DNA-walking method in our laboratory (GenBank number: EF094858). Moreover, it is highly likely that *P. lilacinus* would produce chitinases to degrade the thickest layer of the nematode eggshell (Khan *et al*., 2003). One chitinase gene *PLC* from *P. lilacinus* has also been reported by Dong *et al.* (2007). These proteases enable *P. lilacinus* obtaining nutrients from parasitic nematodes. This capacity likely contributed to the ecological adaptation of this fungus to diverse environments.

In this study, we used sequence data from six loci, including the two virulence-associated genes mentioned above (*PLS* and *PLC*) and 4 housekeeping gene [internal transcribed spacer region of the ribosomal RNA gene (*ITS*), *β-tubulin*, DNA-dependent RNA polymerase II largest subunit (*RPB1*) and DNA-dependent RNA polymerase II second largest subunit (*RPB2*)], to identify nucleotide variation of 80 *P. lilacinus* strains which were collected from 7 different locations in China. These housekeeping genes were chosen because they provide particularly interesting targets for MLST approaches in fungi because a large number of sequences are available in public databases and PCR amplification has been established across most fungal taxa (Lan and Xu, 2006; Fournier *et al*., 2010). The two functional genes were selected because their genetic variations may be different from other non-functional DNA segments and can provide some useful phylogenetically informative characters. Our objective was to reveal the population structure and the reproductive mode of this pathogenic fungus, in the hope of understanding the implications of biocontrol applications in agricultural fields on the conservation and utilization of *P. lilacinus* in nature.

[†]These authors contributed equally to this work.
[*]For correspondence. E-mail: kqzhang111@yahoo.com.cn; Tel.: +86-871-65034878; Fax: +86-871-65034838
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Table 1. **Number of polymorphic sites and haplotype diversity of each gene fragment of *P. lilacinus* we analyzed in this study**

Gene name	Number of nucleotides analysed	Number of polymorphic sites	Number of private haplotypes	Haplotype diversity
PLS	1423	26	8	0.409
PLC	1778	6	6	0.420
ITS	490	36	6	0.479
β-tubulin	412	4	4	0.417
RPB1	443	10	7	0.316
RPB2	886	8	8	0.607
Combined	5432	92	32	0.913

Materials and Methods

Strains and DNA isolation

The 80 isolations of *P. lilacinus* analysed in this study were collected from agricultural soils representing 5-6 main local crops and vegetables (i.e. tomato, cucumber, and soybean) from each of 7 geographical locations in China. Isolation and morphological identification of *P. lilacinus* were based on the method described by Tigano-Milani *et al.* (1995a). These 80 isolates were partly stored in Yunnan Microbiological Fermentation Culture Collection Center (YMF) and partly stored in the Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, P. R. China.

Each strain was maintained on PDA at 28°C for 1 week, and then cultured in the PL-4 liquid medium on a rotary shaker (150 rpm/min) at 28°C for 4 days. Their mycelia were then filtered on a nylon mesh and genomic DNA was isolated using a hexadecyl trimethyl ammonium bromide (CTAB) method (Richards *et al.*, 1994).

Primers, PCR, and DNA sequencing

The selected DNA regions were amplified using the polymerase chain reaction (PCR) from genomic DNA. The primer sequence and the respective expected product size of each primer pair are presented in Supplementary data Table S1. The amplifications were performed using an Eppendorf Mastercycler PERSONAL (Perkin-Elmer, Germany) and the PCR condition used to clone *ITS* and *β-tubulin* genes were same as previously described by Li *et al.* (2005). We also successfully cloned the *RPB1* and *RPB2* fragments using the same protocol. The PCR conditions used to clone *PLS* and *PLC* were as follow: 94°C for 2 min initial denaturation, 32 cycles of 94°C for 40 sec, 60 or 62°C for 40 sec, 72°C for 100 sec; and 72°C for 7 min final extension.

After confirmation of the PCR products by agarose gel electrophoresis, PCR products were cleaned using a Gel Extraction Mini Kit (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd) according to the manufacturer's manual. The purified PCR products were then directly sequenced on both strands using an ABI 3730 autosequencer (Perkin-Elmer, USA) with four fluorescent dyes. Nucleotide sequences were read using the software ChromasPro (www.technelysium.com.au/ChromasPro.html). The six individual gene fragments for each strain were assembled from both strands using Seqman (DNAStar package) after trimming flanking unreadable sequences. Each individual gene sequences from 80 isolates were aligned using ClustalX 1.83 (Thompson *et al.*, 1997) and manual gap adjustments were made to improve the alignment. All six gene fragments which were cloned from the 80 strains were then assembled as a combined dataset.

Data analyses

Population genetic analyses: The individual fragments as well as the combined dataset were imported into program DnaSP Version 5.0 (Librado and Rozas, 2009) to analyze haplotype diversity and identify unique haplotypes, respectively. In both analyses, alignment gaps were all considered as the fifth base.

The local populations were separated according to their geographic sites and grouped together into 2 regions. The analysis of molecular variance (AMOVA) which can estimate the relative contributions of different geographic levels of separation (i.e. local and regional geographic populations) to the overall nucleotide polymorphisms was performed. The Mantel test was used to examine the quantitative relationship between genetic differentiation and geographic distance. The pairwise population genetic distances (Nei's genetic distance) and local geographic distances were calculated between each pairs of populations. The geographic distances between pairs of local populations were calculated directly

Table 2. **Geographic distribution and haplotype diversity of *P. lilacinus* strains we analyzed in this study**

Region	Geographic population (Province)/ collecting site (City)	Crops or vegetables	Sample size	Latitude	Longitude	Haplotype of combined data (number of isolates in each haplotype)	No. of private haplotypes	Haplotype diversity
North	Beijing	cucumber	11	39.51	116.17	18(3), 19(1), 26(1), 27(2), 28(2) ,29(2)	3	0.75
	Heilongjiang/Jiamusi	soybean	14	46.47	130.21	10(1), 11(4), 12(1), 13(1), 14(1), 15(1), 16(1), 17(1), 18(3)	2	0.667
South	Fujian/Changting	tomato	4	25.48	116.06	18(2), 30(1), 31(1)	2	0.667
	Hainan/Sanya	balsam pear	13	18.15	109.32	18(8), 22(1), 23(1), 24(1), 25(2)	0	0.72
	Hainan/Danzhou	pole bean	12	19.24	109.41	18(2), 20(1), 24(9)	6	0.864
	Yunnan/Jianshui	tobacco	12	23.33	102.51	7(1), 9(2), 18(2), 19(1), 20(4), 21(1), 32(1)	1	0.75
	Yunnan/Shilin	tobacco	14	24.45	103.16	1(2), 2(1), 3(2), 4(6), 5(1), 6(1), 8(1)	0	0.75

based on the geographic latitudinal and longitudinal coordinates. Both the AMOVA and the Mantel test were calculated by using the computer program GenAlEx 6.0 package (Peakall and Smouse, 2006).

Phylogenetic analysis: Phylogenetic analyses of individual gene fragments as well as the combined dataset of six loci were performed using the maximum parsimony (MP) method with PAUP* 4.0b8 (Swofford, 2002). A heuristic search strategy was employed with the TBR branch swapping algorithm and 100 replicate random additions. The reliability of tree topology was evaluated using bootstrap support with 1,000 replicates.

Reproduction mode of *P. lilacinus*

Phylogenetically informative polymorphic nucleotide sites were used to identify the modes of reproduction in the *P. lilacinus* strains. For this analysis, each unique haplotype of each gene was counted as a unique allele and the data were then imported to the program MULTILOCUS (Agapow and Burt, 2001). Three complementary tests were conducted: first, the index of association (I_A) was calculated. Statistical significance was derived by comparing the observed dataset to 1,000 simulated datasets against the null hypothesis assuming random recombination. If *P*-value of <0.05 would indicate that the population were clonal reproduction, there would be significant nonrandom association between alleles at different loci (Xu, 2006). Moreover, the I_A value also was standardized by the number of loci with the rBarD algorithm which help facilitate the comparison between populations by adjusting for the numbers of loci (i.e. phylogenetically informative sites). In the second test, the proportion of pairwise loci that were phylogenetically incompatible was calculated. Phylogenetic incompatibility is an indicator of recombination at the population level. The incompatibility ration (IR) can be used as a test for inferences of statistical significance. A *P*-value of less than 0.05 would indicate that the hypothesis of random recombination should be rejected for the population (Agapow and Burt, 2001). In the third test, parsimony tree length permutation test (PTLPT) was used to test for the reproductive mode of *P. lilacinus.* The inability to distinguish between the observed data set and the

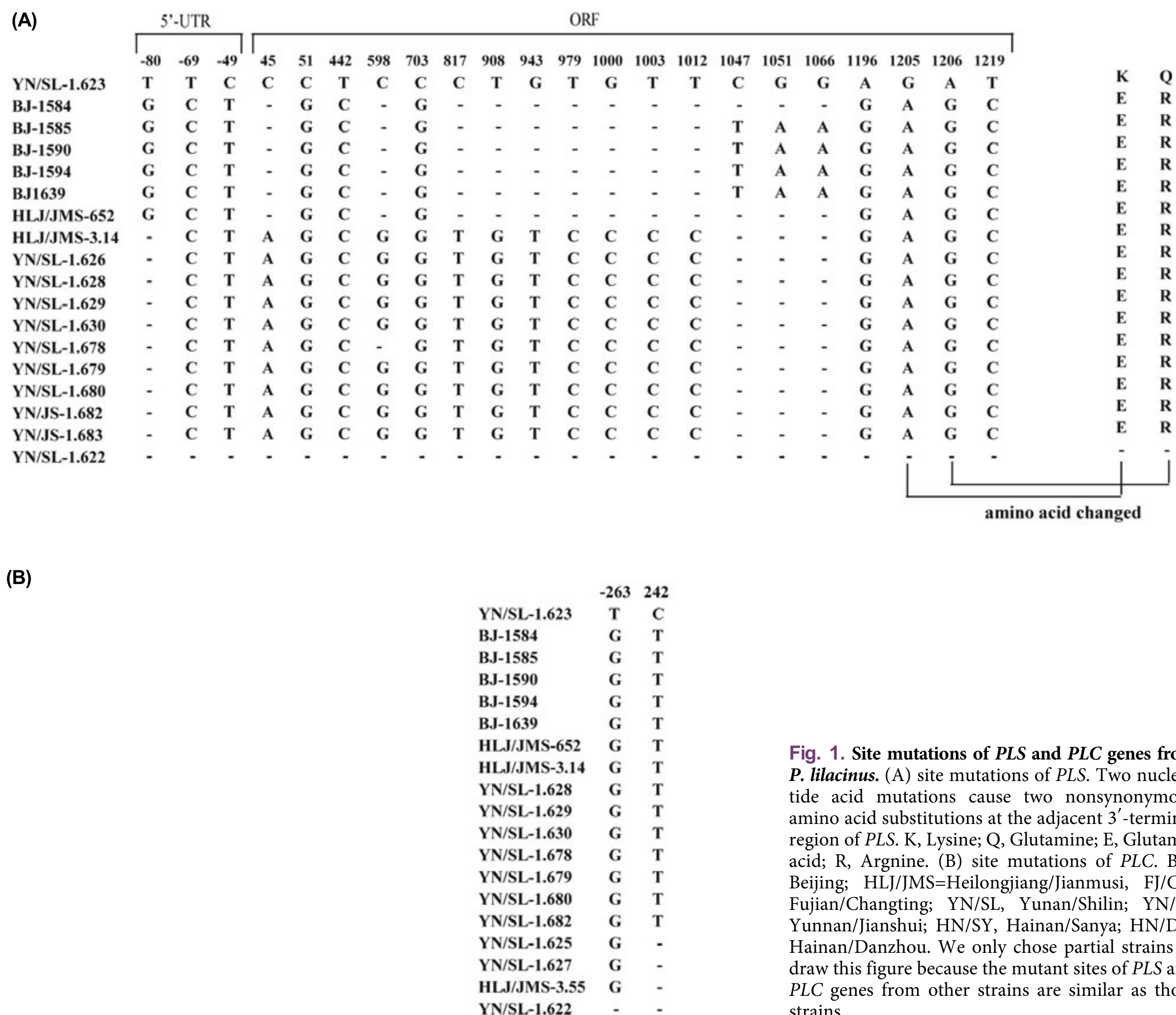

Fig. 1. Site mutations of *PLS* and *PLC* genes from *P. lilacinus.* (A) site mutations of *PLS*. Two nucleotide acid mutations cause two nonsynonymous amino acid substitutions at the adjacent 3′-terminal region of *PLS*. K, Lysine; Q, Glutamine; E, Glutamic acid; R, Argnine. (B) site mutations of *PLC*. BJ= Beijing; HLJ/JMS=Heilongjiang/Jianmusi, FJ/CT, Fujian/Changting; YN/SL, Yunan/Shilin; YN/JS, Yunnan/Jianshui; HN/SY, Hainan/Sanya; HN/DZ, Hainan/Danzhou. We only chose partial strains to draw this figure because the mutant sites of *PLS* and *PLC* genes from other strains are similar as those strains.

Table 3. Analyses of molecular variance (AMOVA) of *P. lilacinus* based on six combined fragments, two virulence-associated genes and four nuclear genes, respectively

Data	Source	Df[a]	SS[b]	MS[c]	Est. Var.[d]	% Var.[e]	AMOVA Statistics	Value	*P* value
Combined	Among Regions	1	5.260	5.260	0.000	0%	PhiRT	-0.014	0.770
	Among Pops	5	28.689	5.738	0.407	25%	PhiPR	0.252	0.010
	Within Pops	73	88.001	1.205	1.205	75%	PhiPT	0.242	0.010
	Total	79	121.950		1.612	100%			
Housekeeping genes	Among Regions	1	4.549	4.549	0.000	0%	PhiRT	-0.002	0.540
	Among Pops	5	21.934	4.387	0.318	27%	PhiPR	0.272	0.010
	Within Pops	73	61.941	0.849	0.849	73%	PhiPT	0.271	0.010
	Total	79	88.425		1.166	100%			
Virulence-associated genes	Among Regions	1	0.710	0.710	0.000	0%	PhiRT	-0.045	0.990
	Among Pops	5	6.755	1.351	0.089	20%	PhiPR	0.200	0.010
	Within Pops	73	26.060	0.357	0.357	80%	PhiPT	0.164	0.010
	Total	79	33.525		0.446	100%			

[a] df, degree of freedom; [b] SS, Sum of squares; [c] MS, Mean squared; [d] Est. Var., Estimated varience; [e] % Var, Percentage of variation.

artificially recombined data sets supports the null hypothesis of sexual recombination, whereas a significant difference between the observed and permuted data sets supports clonality (Burt *et al.*, 1996). In our study, the three tests were conducted on the whole samples based on six loci as well as based on the four housekeeping genes and the two virulence-associated genes (*PLS* and *PLC*).

Results

Population genetic analyses

We successfully amplified and sequenced six gene fragments with totally 5,432-bp nucleotide sites from all 80 strains of *P. lilacinus*, including 3,201 bp from 2 virulence-associated genes (1,423 bp from *PLS* and 1,778 bp from *PLC*, respectively) and 2,231 bp from 4 housekeeping genes (490 bp from *ITS*, 412 bp from β-tubulin, 443 bp from *RPB1* and 886-bp from *RPB2*, respectively) (Table 1). The number of polymorphic sites and haplotypes diversity on each gene fragment and the combined data are presented in Table 1. The combined gene sequences identified 32 unique haplotypes (Table 1). The distribution of individual haplotypes in each geographic population is listed in Table 1. The most common haplotype, h18, contained a total of 20 isolates from 6 of the 7 sites. However, 19 haplotypes each contained only one isolate (Table 2).

For the *PLS* gene, there are 1,423 bp nucleotide sites in total. Sequence alignment of the whole *PLS* gene from 80 *P. lilacinus* strains revealed 22 common point mutations. Three mutations were found at the 5′-UTR and two were C-T transitions and one was G-T transversion. The other 19 point mutations occurred in the ORF of this gene and seven of them were transversions. Furthermore, there were two mutations causing two nonsynonymous amino acid substitutions (Lysine K - Glutamic acid E, Glutamine Q - Argnine R) at the adjacent 3′-terminal region of *PLS* (Fig. 1A).

For the *PLC* gene, there were 1,778 bp nucleotide sites in total. Sequence alignment of the whole *PLC* fragment from 80 *P. lilacinus* strains revealed 2 common point mutations (one was a G-T transversion in the 5′-UTR and one was C-T transition in ORF) (Fig. 1B). Although only a few strains had point mutations in *PLS* and *PLC* genes of *P. lilacinus*, the corresponded mutation site in the mutant strains are conserved (Fig. 1).

A total 92 polymorphic sites among the aligned combined 5,432-bp sequences were used to analyze the genetic structure of the Chinese populations of *P. lilacinus*. The analysis of molecular variance (AMOVA) revealed that 25% of the total genetic variation was due to variation among populations while no significant genetic differentiation was detected among regions (Table 3). The majority of the genetic variance (75%) was found within individual populations. The variance components at the two levels of "among populations" and "within populations" were statistically significant (P=0.01, Table 3). Moreover, the genetic variance based on the four housekeeping genes and the two virulence-associated genes showed similar results: most of genetic variation was found "within individual populations", followed by "between populations within regions" but limited genetic differentiation at the regional level (Table 3).

DNA sequence divergence among populations was estimated using pairwise Nei's genetic distance. The maximum value of 0.581 was found between populations from Yunnan/Shilin and Heilongjiang/Jiamusi while the lowest value was 0.082 between Hainan/Sanya and Yunnan/Jianshui (Table 4). The Mantel test indicated that the relationship between ge-

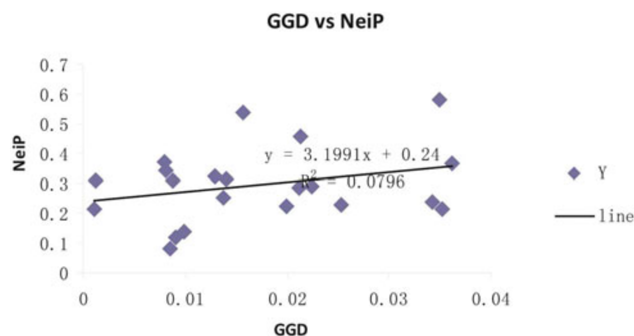

Fig. 2. Relationship of Nei's genetic distances and geographical distances between pairs of local populations of *P. lilacinus* from China (P=0.016).

Table 4. Pairwise population matrix of Nei's genetic distance of *P. lilacinus*

	Nei's genetic distance	data	Pairwise Population Matrix of Nei's Genetic Distance				
	Beijing	Fujian/Changting	Heilongjiang/Jiamusi	Hainan/Sanya	Hainan/Danzhou	Yunnan/Jianshui	Yunnan/Shilin
Beijing	0.000						
Fujian/Changting	0.313	0.000					
Heilongjiang/Jiamusi	0.539	0.227	0.000				
Hainan/Sanya	0.288	0.138	0.216	0.000			
Hainan/Danzhou	0.456	0.121	0.239	0.215	0.000		
Yunnan/Jianshui	0.286	0.253	0.366	**0.082**	0.369	0.000	
Yunnan/Shilin	0.225	0.322	**0.581**	0.308	0.344	0.309	0.000

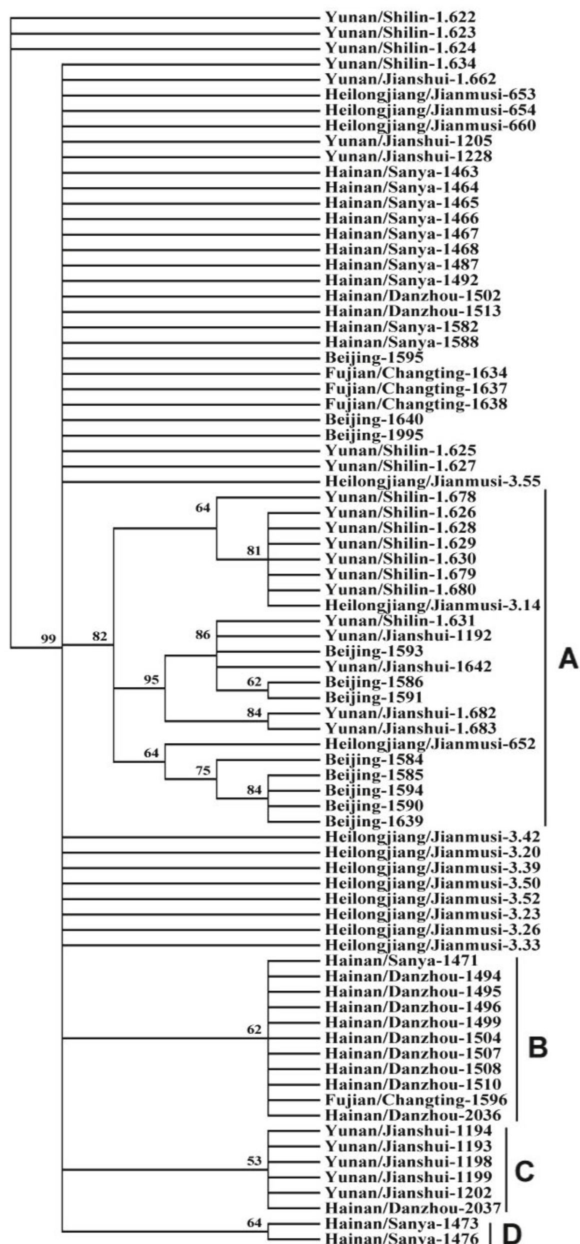

Fig. 3. Phylogenetic tree for 80 *P. lilacinus* isolates generated from the combined dataset. Numbers above lines represent bootstrap values from 1,000 replicates on all parsimony-informative characters, with only bootstrap >50% shown. The figure following each stain number indicates their habitat origins.

netic and geographical distances was positively correlated. However, the correlation was weak and statistically not significant (P=0.06, Fig. 2).

Phylogenetic analysis

The genealogy based on the concatenated six gene fragments were shown in Fig. 3. Although the relationships among many of the 80 strains were not well resolved, four distinct clades which were designated as clades A, B, C, and D were identified (Fig. 3). Clade A falls into three subclades: the first consisted of seven strains from Yunnan/Shilin and one strain from Heilongjiang/Jiamusi; the second contained eight strains from Jianshui and Shilin of Yunnan province and Beijing; the third subclade mainly comprised of strains from Beijing, but also contained one strains from Heilongjiang. Nine strains from Hainan/Danzhou, one from Hainan/Sanya and one from Fujian/Changting were clustered together into clade B. Clade C contains five *P. lilacinus* strains from Yunnan/Jianshui and one from Hainan/Danzhou. Clade D consisted of two isolates from Hainan/Sanya. The phylogenetic trees based on six different gene fragments also formed distinct clades, though the strains in these clades were somewhat different (Fig. 3).

Reproduction mode of *P. lilacinus*

To investigate the reproduction mode of *P. lilacinus*, I_A, RbarD, IR and PTLPT tests were conducted in this study based on six gene fragments using all nucleotide sites, the housekeeping genes and virulence-associated gene fragments, respectively (Table 5). The results identified that I_A, RbarD, and PrC values were consistent with a clonal population structure. Interestingly, for the PTLPT analysis based on all six gene fragments and the four housekeeping genes, the lengths of the most parsimony trees made from the observed data set were significantly different from the distribution of tree lengths calculated for 1,000 randomizations of the observed data set, suggesting the strong ability to reject the null hypothesis of recombination, indicating the clonal population structure for *P. lilacinus*. However, the PTLPT analysis only based on the two virulence-associated genes showed evidence of recombination (Table 5).

Discussion

In this study, we sequenced six gene fragments, including two virulence-associated genes and four housekeeping genes,

Table 5. Multilocus linkage disequilibrium analyses form samples of *P. lilacinus* from China

Analysed gene fragments	I_A[a]	rBarD (*P* value)	PrC[b] (*P* value)	Tree length of PTLPT test		
				Length of observed trees	Lengths of trees based on randomized dataset	*P* value
All six loci	0.637 (P<0.001)	0.128 (P<0.001)	0.600 (P<0.001)	120	202–241	0.010
Four housekeeping genes	0.224 (P<0.001)	0.075 (P<0.001)	0.333 (P<0.001)	66	251–255	0.010
Two virulence genes	0.616 (P<0.001)	0.615 (P<0.001)	1.000 (P<0.001)	32	32	1.000

[a] I_A, The index of association
[b] PrC, the proportion of phylogenetically compatible polymorphic nucleotide sites.

from 80 strains of *P. lilacinus* to analyze natural populations of *P. lilacinus*. Among the six gene fragments, various degrees of nucleotide variation were observed: *RPB2* showed the highest degree of haplotype diversity while *RPB1* showed the lowest (Table 1). The combined dataset identified 32 multilocus genotypes, and nineteen multilocus genotypes contained only one strain each, suggesting some *P. lilacinus* isolates have only limited geographic distribution and might have undergone strong genetic differentiation during their adaptation to environments in different geographic regions. Specifically, like most microorganisms with wide geographical distributions, *P. lilacinus* from different locations also shared the same genotypes, revealing that extensive gene flow have likely occurred among geographic populations of *P. lilacinus* in China. The possibilities of wide dispersion between different geographic areas could be related to the spore dispersal by wind, water or human activities such as transport of agricultural and forestry products. This speculation has been proposed or identified in many fungi (Finlay, 2002; Wang *et al*., 2007; Prospero *et al*., 2008; Sha *et al*., 2008).

Several species in the genus *Paecilomyces* have been reported to have the clear anamorph-teleomorph connections. For example, the first connection of a *Paecilomyces* anamorph with *Byssochlamys* was made by Stolk and Samson (1972). For these thermophilic *Paecilomyces* species, teleomorph associations were assumed to be clavicipitaceous and within the Hypocreales (Samson, 1974) with *Cordyceps* and *Torrubiella* as purported teleomorph connections (Luangsa-ard *et al*., 2004). However, no clear teleomorph has been reported for *P. lilacinus* (Luangsa-ard *et al*., 2004). In our study, three different tests were used to infer the potential reproduction mode of *P. lilacinus*: the I_A test, the IR test and the PTLPT test. Our analyses provided evidence of clonal reproduction mode in natural populations of *P. lilacinus*. We observed that several multilocus genotypes were shared by multiple strains, all from different geographical locations. Specifically, among 32 unique haplotypes identified form the 80 strains of *P. lilacinus*, one haplotype was shared by 20 strains distributed in 6 geographical locations in China (Table 2). Based on the linkage disequilibrium analyses, the alleles from all six combined sequences and also from the virulence-associated genes or housekeeping genes showed no evidence of random association (P<0.001, Table 5). The null hypothesis of random association among the polymorphic nucleotide sites was rejected by the phylogenetic incompatible test (P<0.001, Table 5). The PTLPT test results based on all six combined sequences and the four housekeeping genes both suggests the clonal population structure for *P. lilacinus* (P<0.001, Table 5). Therefore, we believed that the clonal reproduction was the main mode of reproduction in *P. lilacinus*. However, although the index of association test of the two virulence genes *PLS* and *PLC* rejected the null hypothesis of complete random association in our analyses, the PTLPT analysis based on these two virulence-associated genes showed evidence of recombination. Because they both play important roles during the infection of nematode cuticles, we speculated that the two genes *PLC* and *PLS* may be functionally linked and interact with each other epistatically to influence the fitness of strains. Epistatic interactions could allow them to build up of linkage disequilibrium in natural population (Xu, 2004). This may partly explain that why there is little report about the teleomorph connections of *P. lilacinus* in nature. However, it should be emphasized that it cannot exclude the possibility of recombination in *P. lilacinus*. First, our samples were collected from limited geographical regions, the samples analyzed here may not be representative of the global populations of *P. lilacinus*. It is possible that samples from other geographical areas may have evidence of recombination. Therefore, analysis of additional geographical population structure is needed before a more robust conclusion can be drawn about the predominant mode of reproduction in natural populations of *P. lilacinus*. More different marker genes analyses should also give more information about the reproduction mode of this fungus.

Conclusions

The data presented here have implications for the use of *P. lilacinus* as a biocontrol agent of parasitic nematodes. In our analysis, six gene fragments from *P. lilacinus* showed various molecular diversities and there are extensive gene flow might have been occurred among *P. lilacinus* strains in China. The analysis also confirms the clonal reproduction was the main mode of reproduction in *P. lilacinus*. Multilocus analysis of gene fragments may be providing well understands about the population genetic structure of *P. lilacinus.*

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