A.oligospora Strains in China

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Maintaining the effects of nematode-trapping fungi (NTF) agents in order to control plant-parasitic nematodes (PPNs) in different ecological environments has been a major challenge in biological control applications. To achieve such an objective, it is important to understand how populations of the biocontrol agent NTF are geographically and ecologically structured.

Keywords: short tandem repeat ; genetic differentiation ; unique alleles ; non-random recombination

1. Introduction

Plant-parasitic nematodes (PPNs), especially the root-knot nematodes in the genus Meloidogyne, are widespread pests that cause crop yield losses worth more than US $157 billion worldwide each year [1]. For decades, the control of Meloidogyne spp. has heavily relied on chemical nematicides. However, resistance to chemical nematicides has emerged, and the environmental and human health impacts of nematicide residues are becoming increasingly recognized[2]. Therefore, currently available chemical nematicides are being phased out, and an increasing number of biocontrol agents are being introduced to help control PPNs[3].

At present, about 700 fungal species are known to be capable of attacking living nematodes (juveniles, adults, and eggs). These fungi are taxonomically diverse but traditionally divided into five groups based on the mechanisms by which they attack nematodes: (i) nematode-trapping fungi (NTF), (ii) endoparasitic fungi, (iii) egg- and cyst-parasitic fungi, (iv) toxin-producing fungi, and (v) fungi with special nematode-attacking devices [4]. Among these groups, the broad adaptability and flexible lifestyles of NTFs make them ideal agents for controlling PPNs [4][5][6][7]. NTFs are abundantly distributed in a broad range of habitats, especially in temperate agricultural pastures, coniferous leaf litters, and coastal vegetations [8]. Among the NTFs, A. oligospora has generally been considered to be the most common nematode predator in temperate soils [9]. In soil environments, A. oligospora naturally encounters a broad range of nematodes and behaves as a generalist predator with the characteristic ability of forming adhesive trapping nets when its mycelia are in contact with nematodes. Unlike endoparasitic fungi, NTFs are able to grow saprophytically. However, how they maintain and balance their saprophytic and predatory lifestyles are not known. In addition, how geographic populations interact with each other and how ecological factors impact population dynamics remain poorly understood. Such knowledge is important not only for understanding the diversity and evolution of these fungi but also for identifying the most appropriate isolate(s) for commercial biological-control applications [3][6].

Most ecological studies of nematophagous fungi have been restricted to surveys on their geographical and seasonal distributions, including examining the effects of abiotic (e.g., soil conditions) and biotic (mainly nematode density) factors on their distributions [8][9][10][11][12][13][14][15]. Though molecular techniques have increasingly been used to examine phylogenetic relationships among nematode-trapping Orbiliales [16][17][18][19][20]and to investigate the molecular genetics of fungus–nematode interactions [21][22][23][24][25][26][27][28][29], they have scarcely been used to study the patterns of genetic variation present in NTF populations, including the spatial and temporal distributions of genotypes in specific species. The authors of an earlier study[30] analyzed 97 A. oligospora strains from several geographic locations and ecological niches in China using DNA sequences at three gene fragments: its (internal transcribed spacer region of the ribosomal RNA), tub (β-tubulin), and rpb2 (RNA polymerase II subunit). That study identified a large number of unique alleles and genotypes, as well as their limited geographic distributions, consistent with a large effective population size of A. oligospora and its potential for genetic differentiation among geographic populations, likely driven by ecological adaptation. Further analyses of more samples using restriction fragment length polymorphism (RFLP) genotyping revealed a similar pattern . Recently, the heritability of A. oligospora phenotypic variation in the response to nematodes was assessed by analyzing the genetic variation of genomic SNPs using 18 wild isolates. However, due to limited sampling at most geographic locations and/or the small number of molecular loci used, A. oligospora's overall patterns of genetic variations, the relationships between genetic variations and phenotypic variations, and its mode of reproduction remain to be fully described.

2. Genetic Diversity of the Chinese Samples of A. Oligospora Detected by STRs and MLST

Based on 200 initially designed STR primer pairs from the genome of *A. oligospora* (ATCC 24927), we selected 20 to genotype strains of *A. oligospora.* A previous study classified the polymorphism level of STR markers based on PIC values into low (PIC < 0.25), medium (0.5 > PIC > 0.25), and high (PIC > 0.5) [51]. Based on this grouping, the 20 STR markers we developed for *A. oligospora* were found to have a moderate to high level of polymorphism, with 13 having high polymorphism, six having medium polymorphism, and only one having a low PIC value. The highest discriminatory power value for a single locus was obtained with A191, with a gene diversity value of 0.87 and 21 different alleles in our sample.

Among the 239 *A. oligospora* isolates from 19 different geographical populations, a total of 188 alleles and 178 multilocus genotypes were found based on the 20 STR loci. The number of alleles range from 3 to 21, with an average of 9.4 alleles per locus. Of the 188 alleles, 140 were shared between at least two of the 19 geographical populations in China, the remain 48 alleles were found only in one geographical population each. The 19 geographical populations also differed in their total numbers of alleles, which ranged from 21 (Kanas Lake, Xinjiang) to 88 (Heijing, Yunnan). Except for their absence in three geographic populations (Inner Mongolia, Qinghai, and Kanas Lake, and Xinjiang populations), private alleles were found in all other 16 populations. Of the total 178 STR multilocus genotypes, only four were shared by two or more geographical populations, and the other 174 were only found in one geographical population each. The most shared genotype was found among two populations from Xinjiang and three populations from Dianchi, Yunnan, Sichuan, and Hubei.

3. Genetic Differentiation and Population Structure

Though significant genetic differentiations were found among many geographic populations, our analyses also showed evidence of gene flow among certain geographic populations. The existence of gene flow and genetic differentiation was further supported by results from the STRUCTURE analysis that showed that there were two genetic clusters (K = 2) in the Chinese population of *A. oligospora*, and most geographic populations contained alleles and genotypes of both genetic clusters. The population structure indicated by STRUCTURE software and PCoA was very similar, and both the MLST and STR datasets identified two distinct genetic clusters in all 239 *A. oligospora* isolates. Specifically, most samples from Southwestern China (Sichuan, Guizhou, Tibet, and Yunnan including Dianchi, Heijing, Yimen, and Gejiu) and Hainan Island formed one cluster, while those from other parts of China formed another cluster. Populations in the former clade were found to have more frequent genetic exchanges than those in the second cluster. However, the composition of two different genetic clusters indicated by the two kinds of molecular markers were quite different in some geographic populations. For example, STRs detected the existence of genetic elements of the second cluster in geographic populations from Hubei, Inner Mongolia, Jilin, Xinjiang, and Guangxi, while they were absent in the MLST dataset. Interestingly, most genotypes containing elements of both genetic clusters were distributed in Southwestern China and Hainan. These genotypes likely represent hybrids of those two genetic clusters.

4. Phylogenetic Divergence

Phylogenetic analyses based on SNPs and MLST identified two distinct clades (Ⅰ and Ⅱ) within *A. oligospora* from 19 geographic populations in China, consistent with the two genetic clusters revealed by the population structure analyses. Clades Ⅰ and Ⅱ of the MLST phylogeny included 38 and 21 multilocus sequence types, respectively. Most *A. oligospora* samples from different geographic populations (except for Guizhou) were widely distributed across Clade ⅠI. Interestingly, different geographical populations had different distribution patterns between the two clades: five geographic populations (Hubei, Jilin, Guangxi, and two populations from Xinjiang) were only found in Clade ⅠI, and samples from Tibet were only found in Clade Ⅰ. Two populations (Hainan and Heijing from Yunnan) were widely distributed in both clades.

Significant differences in the relationships among strains among the analyzed gene fragments were identified based on the pairwise comparisons of topologies among all single gene phylogenetic trees (Figures 1). The results indicated evidence of potential allele-sharing and/or hybridization between the genetic lineages of this important NTF. For example, strains containing both blue and red alleles in the STRUCTURE analysis (colored in green on trees) formed tight and independent clusters on trees of *mapk*, *rpb2* and *sp* fragments, suggesting they belonged to populations that are diverging from the other two clades. However, the same strains had mixed relationships with others based on sequences at *its*, *tub,* and *tef-1* loci. Together, these differences in phylogenetic relationships among strains by different gene fragments are consistent with recombination in nature.

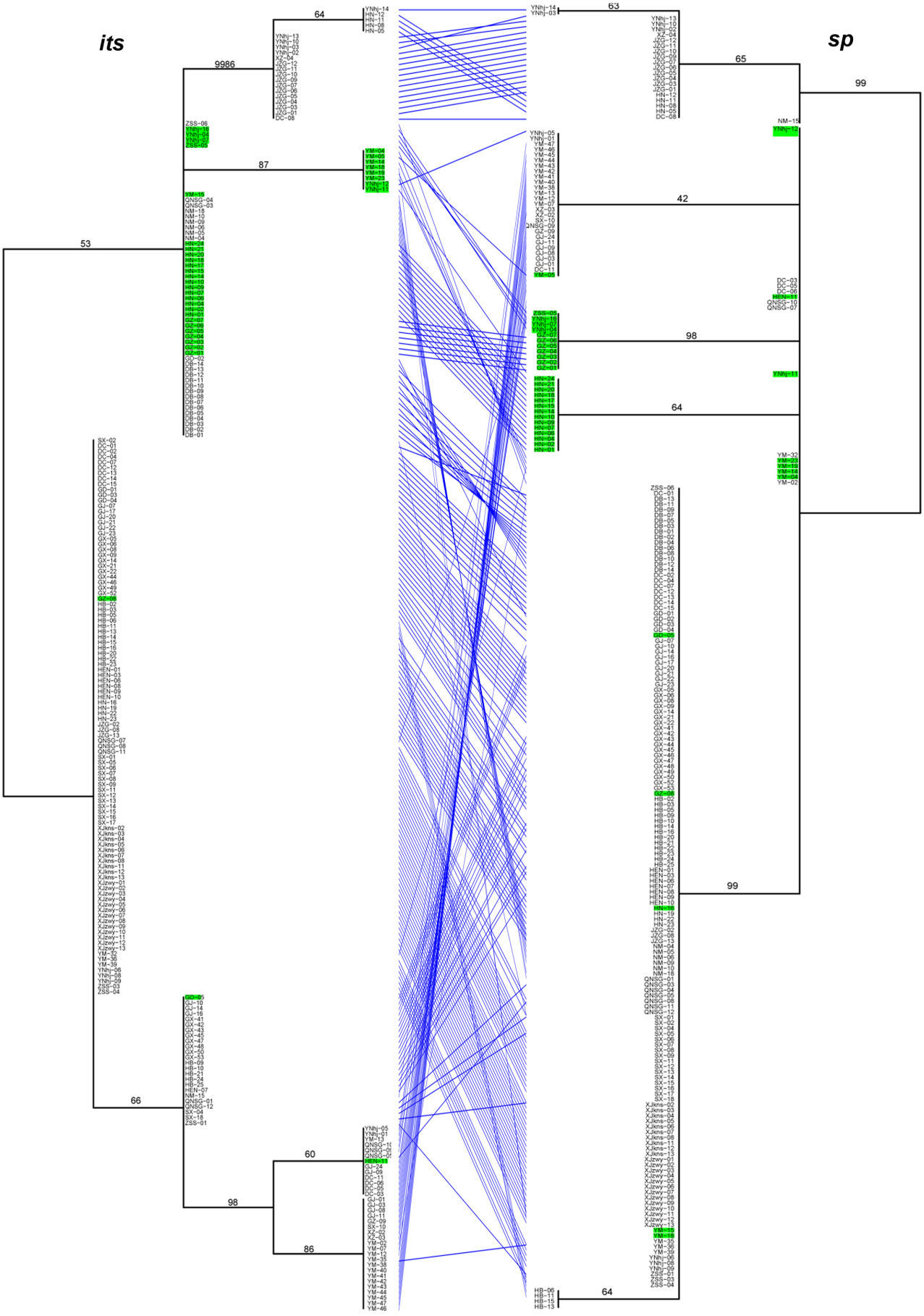

Figure 1. The tanglegram between *its* and *sp* phylogenies. Notes: Branch support values are indicated by numbers near branches, and putative hybrids (as suggested by STRUCTURE analysis) are colored in green in the tanglegram.

5. Clonality and Recombination

The rBarD and phylogenetic incompatibility tests for detecting recombination were conducted for (i) the total sample, (ii) samples from each geographical sample groups by both the STR and MLST datasets, and (iii) samples representing MLST genotypes in each of the phylogenetic clades (Table 1); the allelic sequences at each of the six MLST loci were treated as alleles in these tests. Though predominantly clonal population structures were detected for these sample sets,

we found unambiguous evidences for recombination among the 20 STR loci (PrC = 0.01, $p < 0.001$; rBarD = 0.355, $p < 0.001$) and six MLST loci (PrC = 0, $p = 1$; rBarD = 0.6398, $p < 0.001$) in the total sample, including all 239 *A. oligospora* isolates. As indicated by the phylogenetic incompatibility test, variable levels of recombination were found within different sample groups. Interestingly, population groups from Southwestern China had the highest levels of phylogenetic incompatibility. It is worth noting that phylogenetic incompatibility was also identified in all samples where random recombination was rejected, indicating that low levels of recombination are common in samples of *A. oligospora* in China.

Table 1. Results of multilocus linkage disequilibrium analyses for different sample groups of *A. oligospora* from China.

Sample Groups	Sample Size	Phylogenetic Compatibility (*p* Value)		rBarD (*p* Value)	
		MLST	STR	MLST	STR
Total	239	0 (1)	0.01 (<0.001)	0.6398 (<0.001)	0.355 (<0.001)
Central	25	0.8667 (0.447)	0.7 (0.038)	0.1077 (0.009)	0.1331 (<0.001)
East	5	1 (1)	0.9947 (0.096)	0.6805 (0.001)	0.3039 (<0.001)
North	24	0.9333 (1)	0.8 (<0.001)	0.2784 (<0.001)	0.2772 (<0.001)
Northeast	14	1 (1)	0.9211 (0.009)	-nan * (<0.001)	0.115 (<0.001)
Northwest	33	0.8 (0.889)	0.8632 (<0.001)	0.576 (<0.001)	0.3988 (<0.001)
South	46	0.4667 (<0.001)	0.2526 (<0.001)	0.6867 (<0.001)	0.288 (<0.001)
Southwest	92	0.2667 (<0.001)	0.0579 (<0.001)	0.661 (<0.001)	0.3874 (<0.001)
Clade I	151	0.1333 (<0.001)	0.021 (<0.001)	0.5042 (<0.001)	0.2019 (<0.001)
Clade II	46	0.5333 (<0.001)	0.3052 (<0.001)	0.6379 (<0.001)	0.4439 (<0.001)

Notes: Geographic populations included in each of the major regions in China: Central: Hubei, Henan; East: Zhejiang, Shandong; North: Hebei, Nei Mongol; Northeast: Jilin; Northwest: Shaanxi, Qinghai, Xinjiang; South: Guangdong, Guangxi, Hainan; Southwest: Sichuan, Guizhou, Yunnan, Tibet. * All strains in this population have the same haplotypes in six gene fragments. MLST: multilocus sequence typing; STR: short tandem repeat; rBarD: standardized index of association.

6. Phenotypical Characterization

6.1. Conidial Morphology

For conidial morphology analyses, 38 strains of *A. oligospora*—including 30, 6, and 2 strains from the divergent Clades A, B, and C identified in our previous study [30], respectively—were selected to observe. Clades A and B respectively correspond to our Clades Ⅰ and Ⅱ here, and Clade C falls into the basal branch of Clade Ⅱ. After conidia sporulation on CMA, the length and width of 50 mature conidia of each strain were observed and measured, and the length–width (L/W) ratio was selected as the quantitative measure for the following statistical analyses.

First, we tested variance homogeneity among the data from three different phylogenetic clusters. A Levene's p value of greater than 0.05 was obtained, suggesting there was no significant difference in the total variance among the three clusters. Second, an analysis of variance was conducted. The L/W ratio of Clade C strains was found to be significantly

higher than those of Clades A and B by Duncan's multiple comparison test (Figure 2c). The L/W ratios of strains from Clades A and B were not significantly different from each other. Together, our results indicated some morphological differentiation among the clades within *A. oligospora* strains from China.

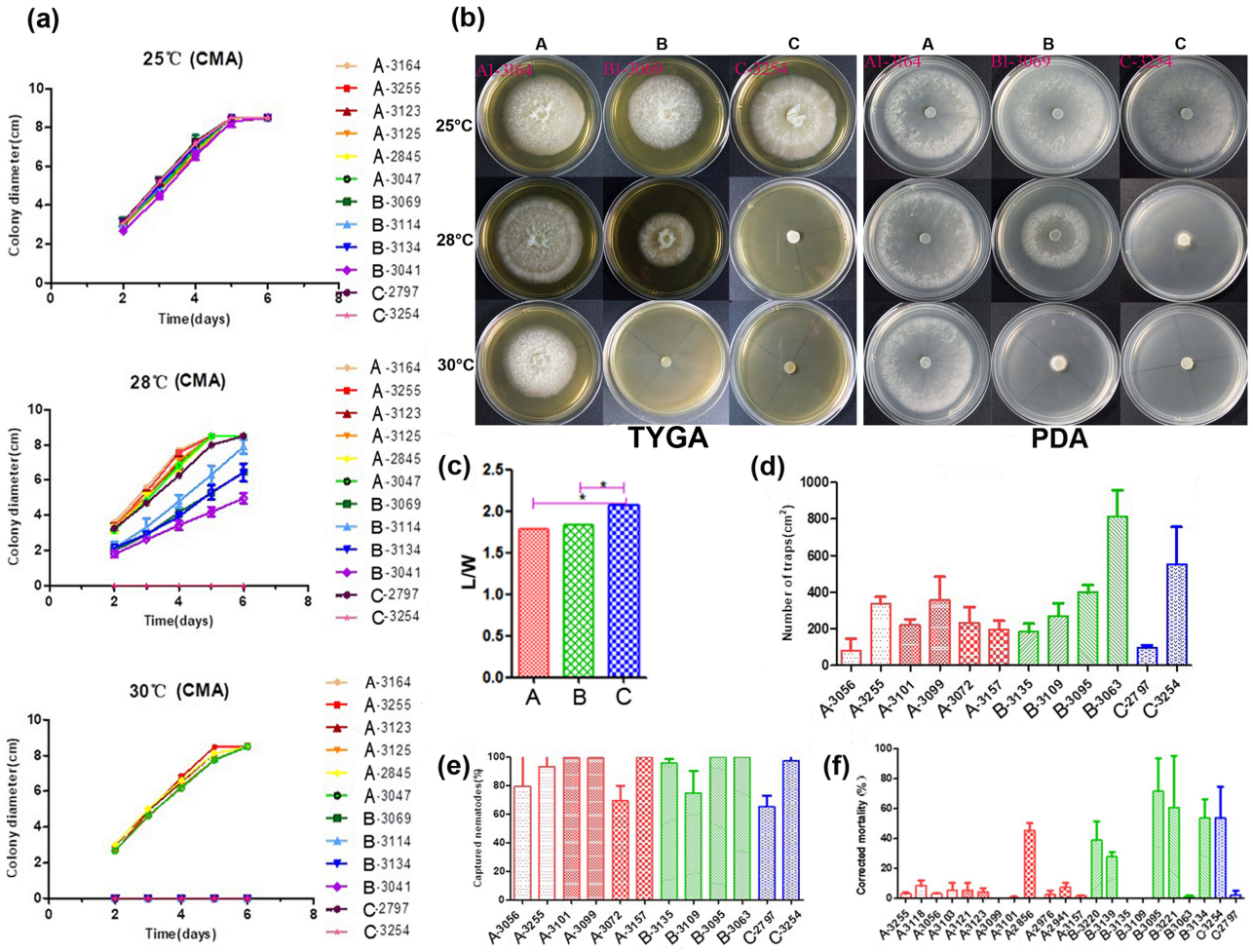

Figure 2. Phenotypical characterization of strains from the three main lineages. (**a**) The growth state of 12 representative *A. oligospora* strains incubation at 25, 28, and 30 °C on CMA. (**b**) Colony morphology of representative strains at 25, 28, and 30 °C on TYGA and PDA plates. (**c**) Pairwise comparisons of L/M among representative strains. (**d**) Comparison of trap formation, (**e**) captured nematodes, and (**f**) nematicidal activities of fermentation broth among representative strains. Notes: CMA: corn meal agar, TYGA: the tryptone, yeast extract, and glucose agar, PDA: potato dextrose agar, * significantly different.

6.2. Mycelial Growth

A total of 12 strains representing three clades (six from Clade A, four from Clade B, and two from Clade C) from different geographic populations were selected to investigate their potential differences in mycelial growth rate and colony morphology on three different media at 25, 28, and 30 °C. The fungal colonies on the CMA medium were generally very loose and their aerial hyphae were sparse. By contrast, those on TYGA medium produced extremely dense mycelia and their aerial mycelia grew most robustly. The growth status of hyphae on the PDA medium was intermediate between CMA and TYGA (Figure 2b).

Overall, the optimal growth temperature of different *A. oligospora* strains was 25 °C. At 25 °C, all strains could grow normally and showed the fastest growth rate among the three tested temperatures. However, the growth rates of isolates from Clades B and C (except YMF1.02797 from Clade C) were slower than those of Clade A at 28 °C, and the aerial mycelia deviated from radial spread. In fact, isolates from Clades B and C were unable to grow at 30 °C (Figure 2a,b). Together, these results suggest clade-specific differences in colony morphology and mycelial growth, regardless of geographic origins.

6.3. Conidial Yield, Trap Formation and Nematode-Trapping Bioassay

A. oligospora, which can capture nematodes by forming three-dimensional networks from specialized hyphae, is considered the most abundant nematode predator in temperate soils [11]. In addition, genetic differences have been reported to have effects on the response of *A. oligospora* to its prey, though robust correlation has not been observed [21].

Therefore, the conidial yield, trap formation, and nematode-trapping bioassays were further investigated to examine whether there were notable differences among nematicidal activities among the three phylogenetic clades identified in a previous study.

Overall, we found significant differences in conidial yield among strains, but the differences among phylogenetic clades were not statistically significant. Strains from each of the phylogenetic clades have variable abilities to produce conidia. For example, three strains from the three clades (with one representing each clade)—YMF1.03101 (Clade A), YMF1.03135 (Clade B) and YMF1.03254 (Clade C)—were found to have significantly higher conidial yields than all other tested strains from either the same or different clades.

The number of traps produced by different strains of *A. oligospora* was most distinguishing when exposed to *C. elegans* for 12 h. The highest number was produced by strain YMF1.03063 of Clade B at >47% more than others, while the numbers produced by YMF1.03056 of Clade A and YMF1.02797 of Clade C were the least, only accounting for about 20% of that produced by YMF1.03063. The number of trappers produced by other tested strains were similar (Figure 2d). Overall, there was no significant correlation between the various abilities of the trap formation and phylogenetic division of *A. oligospora* strains from China. Correspondingly, limited difference regarding nematode-trapping abilities was found among the three phylogenetic clusters (Figure 2e).

We further detected the pathogenicity of fermentation broth from different representative strains in the three phylogenetic clades. Forty-eight hours after the nematodes were added into the fermentation supernatants, the lethal effects of different *A. oligospora* strains on nematodes was significantly different. Generally, strains YMF1.02856 (Clade A), YMF1.03095 (Clade B), and YMF1.03254 (Clade C) had obvious nematicidal effect, and the corrected mortality caused by strain YMF1.03095 was as high as 71.5%. However, strains YMF1.03056 and YMF1.02797, which showed the distinguishing ability to form traps and capture nematodes on the solid media, produced fermentation supernatants with weak pathogenicity (Figure 2f). Overall, we found no significant difference among clades in terms of the nematicidal activity of their fermentation broth.

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