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Intraspecific Variability among the Isolates of *Metarhizium anisopliae* var. *anisopliae* by RAPD Markers

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Abstract

Insect fungal pathogens embrace an extraordinary position in microbial pathogenesis. For selection of effective species for the insect management and for considering the factors that alter the natural epizootics, it is vital to understand the phylogenetics of the particular species present in an ecosystem. The genus *Metarhizium* comprises of identical species which are present in the ecosystem for the management of number of insect pests from different insect orders. For the use of fungi as microbial control agents, knowledge of the genetic stability of the released strain is indispensable. For this purpose DNA polymorphism among 62 isolates of the entomopathogenic fungus *Metarhizium anisopliae* var. *ansiopliae* isolated from diverse soil ecosystems was studied by RAPD-PCR. DNA fragments were achieved between 150-1400bp using ten 10-mer PCR primers of random nucleotide sequence. The results showed each isolate differed in the size and number of RAPD products, indicating significant polymorphism. The primer OPA-04 showed imperative results by producing high number of polymorphic bands. The similarity data dendogram clustered the isolates into three main clades on the basis of their geographical origins with a common average similarity coefficient of 45.3%.The analysis of molecular variance results showed that the variation within the population was 14.35%.

Keywords: Variability; RAPD; Genomic DNA; *Metarhizium anisopliae*; Insect pathogenic fungi

Introduction

The utilization of entomogenous microbes to manage insect pests is presently deemed to be a possible substitute to the conventional insecticides, which are notorious to have noxious effects on the beneficial organisms (St. Leger *et al.,* 1996). In contrast to biological control means, fungi need no ingestion for host infection and can enter the cuticular layer, therefore can, be used efficiently for the management of majority of insect pests. Insect pathogenic fungus *Metarhizium anisopliae* has great potential for biological control of insect pests (Gillespie and Claydon, 1989; Heale *et al.,* 1989; Akbar *et al.,* 2012). With rational strain improvement it becomes feasible to understand the biochemical and molecular basis of pathogenicity (Clarkson, 1992). *M. anisopliae* has a wide host range infecting more than 200 insect pest species (Zimmermann, 1993; Freed *et al.,* 2012a, b; Akmal *et al.,* 2013).

The release of a bio control agent need the demonstration of its capacity to identity and examine its effect on the target pest (Bidochka *et al.,* 2001). Additionaly it involves the separation of the organism from the local populations. Conventionally, classification of *Metarhizium* is based on the morphological features (Bridge *et al.,* 1997; Driver *et al.,* 2000). Complexities in classification commonly occur as different morphological characters (e.g., variable size and shape of conidia and blastospores) that can be present in the similar organism under changeable conditions (Glare *et al.*, 1996).

The genetic associations in the genus *Metarhizium* have been checked through various molecular methods e.g. Restriction Fragment Length Polymorphism (RFLP) (Bridge *et al.,* 1993; Pipe *et al.,* 1995), comparison of r-DNA sequence data (Rakotonirainy *et al*., 1994; Freed *et al.,* 2011a) and Random Amplification Polymorphic DNA (RAPD) (Cobb and Clarkson, 1993; Bidochka *et al*., 1994; Leal *et al*., 1994). These methods have however only been partially successful in providing a technique suitable for strain identification. RAPD-PCR technique which is generally considered as a powerful diagnostic tool for differentiation has shown intraspecific variation (Velásquez *et al*., 2007). Presently, simple sequence repeats markers have been used as powerful markers due to their highly polymorphic nature for studying the phylogenetics of insect pathogenic fungi (Coates *et al.*, 2002; Enkerli *et al.*, 2001; 2005; Freed *et al.,* 2011a).

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The aim of current study was to define the level of genetic variability of *M. anisopliae* from different regions i.e., China, South Korea, Laos, Singapore and The Netherlands by using RAPD and to assess most suitable markers for the analysis of genetic variability of this entomopathogen.

Materials and Methods

Isolation and Maintenance of Entomogenous Fungal Isolates

A wide range of environments including, agricultural, forest and urban environment were selected from China, South Korea, Laos, Singapore and The Netherlands for the collection of samples (Figs. 1-3). Soil samples were taken from 10 cm deep under the earth from different provinces of the above described countries. After the collection of the samples, these were preserved at 4°C until used for isolation of *M. anisopliae*. The collected samples were used to isolate *M. anisopliae* on semi-selective culture media as previously done by Veen and Ferron (1966) and Freed *et al.* (2011a, b).

All isolates (Table 1) were consequently cultivated from a solitary conidium. For this, 100 µL of a suspension with a low concentration of conidia in phosphate buffer was extended on potato dextrose agar (PDA) plates. After 24 h, a single germinated conidium was spotted by a microscope, a portion of the media representing merely marked conidium was excised and shifted to PDA, and these plates were retained at 25°C. The morphological characters of *Metarhizium* were verified by microscope after 5 days of growth. The 14 days old spore were later on harvested and preserved in sterile 30% glycerol at -20°C for experimentation.

Genomic DNA Isolation

The genomic DNA was extracted by the method described by (Liu *et al.,* 2000; Freed *et al.,* 2011a, b). The resultant DNA was electrophoresed in 1% TBE-agarose gel (Invitrogen Corp., Burlington, ON), stained with EtBr, and seen below ultra violet light. The DNA was purified by Gel Extraction Kit (OMEGA, USA). The purified DNA was used for the PCR amplification.

RAPD Amplification

For all isolates, RAPD-PCR of purified DNA of each isolate was executed in a Bioer XP-cycler for 40 cycles of 35 s at 94°C, 35 s at 40°C and 1 min at 72°C, and a final additional 10 min cycle at 72°C. PCR reactions were performed in an overall quantity of 25 µL containing 1 x PCR amplification buffer, 0.3 mM of $MgCl_2$, 0.2 µM of dNTPs, 0.2 µM of the primer, 1 U *Taq* DNA Polymerase (Takara), 2.5 µL (20-25 ng) of purified DNA and ultra pure

Fig. 1: Map of China showing the sampling sites for soil samples collection

Fig. 2: Map of Laos showing the sampling sites for soil samples collection

Fig. 3: Map of Netherlands showing the sampling sites for soil samples collection

Table 1: *Metarhizium anisopliae* var. *anisopliae* isolates from different geographical origin and source

S. No.	Code	Origin	City/Province	Source	S. No.	Code	Origin	City/Province	Source
1.	CNCG	P.R. China	Zhongxian, Chongqing	Agricultural soil	32.	CNYN4	P. R. China	Libo, Yunan	Forest soil
2.	CNGD1	P.R. China	Zhuhai, Guangdong	Agricultural soil	33.	CNZH	P. R. China	Hangzhou, Zhejiang	Forest soil
3.	CNGD2	P.R. China	Zhaoqing, Guangdong	Orchard soil	34.	CNXJ3	P. R. China	Tekesi, Xinjiang	Forest soil
4.	CNGD3	P.R. China	Yingde, Guangdong	Agricultural soil	35.	CNXJ4	P. R. China	Wen Xuxian, Xinjiang	Agricultural soil
5.	CNGD4	P.R. China	Cheping, Guangdong	Agricultural soil	36.	CNXJ5	P. R. China	Ku erle Shi, Xinjiang	Peer Orchard soil
6.	CNGD5	P.R. China	Kaiping, Guangdong	Orchard soil	37.	CNXJ6	P. R. China	Ba Chuxian, Xinjiang	Forest soil
7.	CNGD6	P.R. China	Maoming, Guangdong	Agricultural soil	38.	CNXJ7	P. R. China	Wen Quanxian, Xinjiang	Sunflower field
8.	CNGD7	P.R. China	Shaoguan, Guangdong	Agricultural soil	39.	LSHY	Laos	Houayin, Champasak	Forest soil
9.	CNGD8	P.R. China	Huizhou, Guangdong	Forest soil	40.	LSNA1	Laos	Namphao, Bolikhamxai	Forest soil
10.	CNGD9	P.R. China	Yang jiang, Guangdong	Forest soil	41.	LSNA2	Laos	Namphao, Bolikhamxai	Forest soil
11.	CNGD10	P.R. China	Huizhou, Guangdong	Forest soil	42.	LSNM1	Laos	Namsanam, Khammouane	Forest soil
12.	CNGD11	P.R. China	Yang Jiang, Guangdong	Orchard soil	43.	LSNM2	Laos	Namsanam, Khammouane	Forest soil
13.	CNGU1	P.R. China	Leishan, Guizhou	Forest soil	44.	LSNM3	Laos	Namsanam, Khammouane	Forest soil
14.	CNGU2	P.R. China	Taiba, Guizhou	Mountain soil	45.	LSPK	Laos	Khouay NBCA, Vientiane Prefecture	Forest soil
15.	CNGU3	P.R. China	Leishan, Guizhou	Forest soil	46.	LSTC1	Laos	Tadchampa, Champasak	Forest soil
16.	CNGX	P.R. China	Dongxing, Guangxi	Forest soil	47.	LSTC2	Laos	Tadchampa, Champasak	Forest soil
17.	CNHB1	P.R. China	Shennogjia, Hubei	Forest soil	48.	LSTC3	Laos	Tadchampa, Champasak	Forest soil
18.	CNHB2	P.R. China	Shennogjia, Hubei	Forest soil	49.	LSVT1	Laos	Vientiane	Forest soil
19.	CNHB3	P.R. China	Shennogjia, Hubei	Forest soil	50.	LSVT2	Laos	Vientiane	Forest soil
20.	CNHE	P.R. China	Linzhou, Henan	Mountain soil	51.	LSVY	Laos	Viengxay, Houaphan	Forest soil
21.	CNHN1	P.R. China	Lingshui, Hainan	Forest soil	52.	NLAN	Netherlands	Arnhem, Gelderland	Potato field soil
22.	CNHN2	P.R. China	Baoling, Hainan	Forest soil	53.	NLHN2	Netherlands	Heteren, Gelderland	Vegetable field soil
23.	CNHN3	P.R. China	Lingshui, Hainan	Forest soil	54.	NLHN3	Netherlands	Heteren, Gelderland	Corn field soil
24.	CNHN4	P.R. China	Lingshui, Hainan	Forest soil	55.	NLUT	Netherlands	Utrecht	Urban land soil
25.	CNSN1	P.R. China	Dujiang Yan, Sichuan	Forest soil	56.	NLWN	Netherlands	Wageningen, Gelderland	Grass field soil
26.	CNSN2	P.R. China	Baoxing, Sichuan	Cicadas	57.	SKCJ1	South Korea	Cheju island	Volcano soil
27.	CNXJ1	P.R. China	Hami, Xinjiang	Agricultural soil	58.	SKCJ2	South Korea	Cheju island	Urban land soil
28.	CNXJ2	P.R. China	Hami, Xinjiang	Agricultural soil	59.	SKCJ3	South Korea	Cheju island	Urban land soil
29.	CNXZ	P.R. China	Linzhi, Tibet	Agricultural soil	60.	SKCJ5	South Korea	Cheju island	Volcano soil
30.	CNYN1	P.R. China	Pingbian, Yunan	Forest soil	61.	SPCT1	Singapore	Singapore city	Urban soil
31.	CNYN2	P.R. China	Yingjiang, Yunan	Forest soil	62.	SPCT2	Singapore	Singapore city	Urban soil

water. PCR reactions were repeated twice to assess the uniformity of the band for the isolates. The resultant PCR products were separated on a 1.5% agarose in 1× TAE at 100 V for 2 h. The agarose gels were stained with ethidium bromide and visualized with UV light and photographed for the estimation of variability. A pre-selection of five isolates of *M. anisopliae* with 50 primers from the OPA-A, OPA-B, OPA-C, OPA-AD and OPA-AB series (Operon Technologies, California, USA) was executed to choose the most useful primers. For the experimentation of the 62 isolates we selected 10 primers (*OPA*-03, 04, 09, 10, 12; *OPC*-01, 04, and *OPAB*-03, 04, 09) (Table 2).

Data Analysis

For each RAPD primer, polymorphic bands appeared to have dual characters and were calculated as present (1) or absent (0). Genetic likeness amongst pairs was assessed using Jaccard's coefficient. The likeness matrix was calculated on sequential, agglomerative, hierarchical and nested clustering (SAHN) (Sneath and Sokal, 1973) using the neighbor joining (NJ) clustering algorithm to generate a dendogram. Analysis of molecular variance (AMOVA) was computed to simplify molecular models of intraspecific difference for the 62 isolates (Excoffier *et al.*, 2005). All calculations were accomplished by means of Arlequin 3.01. The total variation was segregated into genetic variance between/ among ecological locations and among isolates within different ecological locations.

Results

Ten RAPD primers used to analyze 62 isolates of *M. anisopliae* produced an aggregate of 105 bands ranging from 150-1400bp, but the bands which amplified constantly over two DNA extractions and PCR experiments were utilized for the investigation. It was recorded that 95.65 bands were polymorphic and 2-13 bands were obtained for each marker, with an average of 7.4 bands per marker (Figs. 4-7). The results indicate that out of 10 primers 5 primers (*OPA-04*, *OPA-03*, *OPA-10*, *OPAB-09*, and *OPA-12*) were most instructive, since these identified the maximum polymorphic bands and generated a high number of band patterns, out of which 49.45% bands were polymorphic. The primer OPA-04 was imperative because it gave high number of polymorphic bands. The similarity data dendogram clustered the 62 isolates into three main clades with a common average similarity coefficient of 45.3%. The clade I and II clustered mainly the isolates from China, Laos, Netherlands and South Korea,

Table 2: RAPD primers sequence and characteristics

Primer	Sequence (5'-3')	Annealing Temp. (°C)	Reference
OPA-03	AGTCAGCCAC	39	Manfredi *et al.,* 2006
OPA-04	AATCGGGCTG	39	Cobb and Clarkson, 1993
OPA-09	GGGTAACGCC	39	Cobb and Clarkson, 1993
OPA-10	GTGATCGCAG	39	Cobb and Clarkson, 1993
OPA-12	TCGGCGATAG	39	Singh *et al.,* 2006
OPC-01	TTCGAGCCAT	39	Vastag *et al.,* 2000
OPC-04	CCGCATCTAC	39	Vastag *et al.,* 2000
OPAB-03	TGGCGCACAC	39	Chakraborty and Sikdar, 2008
OPAB-04	GGCACGCGTT	39	Chakraborty and Sikdar, 2008
OPAB-09	GGGCGACTAC	39	Chakraborty and Sikdar, 2008

Table 3: Analysis of molecular variance (AMOVA) results and fixation index (F_{ST} θ) of the random amplified polymorphic DNA (RAPD) for the isolates of *M. anisopliae* var. *anisopliae*

Source of variation	df	Sum of squares	Variance components	Percent variation
RAPD				
Among populations	1	99.237	3.5133va	14.35
Within populations	61	1341.215	33.0310vb	85.65
Total	62	1440.452		
Fixation index	F_{ST}	0.1502		

while clade III contained majority of the isolates from China, while one isolate i.e., LSVY and NLHN3 each from Laos and Netherlands (Fig. 8). The results showed practicality of the RAPD-PCR analysis in perceiving the genetic variance within and among groups of isolates assembled from different ecological areas.

The analysis of molecular variance results for RAPD data specify that the variation within population was 85.65% of the total variation, while the difference between populations was stated to be 14.35%. The fixation index (F_{ST} = 0.1502) showed little differentiation had occurred among the samples of *M. anisopliae* (Table 3).

Discussion

Genetic differences in the insect pathogenic fungi have been evaluated by means of various markers to describe various strains and to know of the host and microbes associations (Maurer *et al.,* 1997). The studies indicate host variety to be the major feature influencing population while Maurer *et al.* (1997) reported a grouping which showed insect and pathogen interaction without focusing the geographical origin. Additional aspects may also be engaged as a confirmation by the occurrence of isolates which didn't cluster within the same insect and pathogen groups. It is proposed that an enhanced relationship among genotypes and host range could be done on the availability of information regarding the genetic variability associated within a given host species.

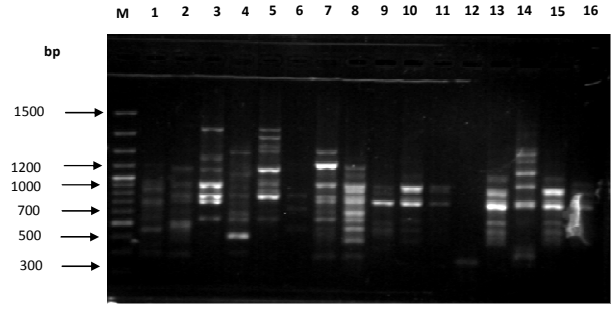

Fig. 4: RAPD marker *OPA-04* showing the banding pattern of the isolates of *M. anisopliae* var. *anisopliae* (1-16 in order), CNHB3, LSVT2, CNGD3, CNGD5, CNYN4, CNHB1, CNGU1, NLUT, NLWN, CNZH, CNXZ, SKCJ1, LSHY, CNGD8, CNGU2 and CNHN1

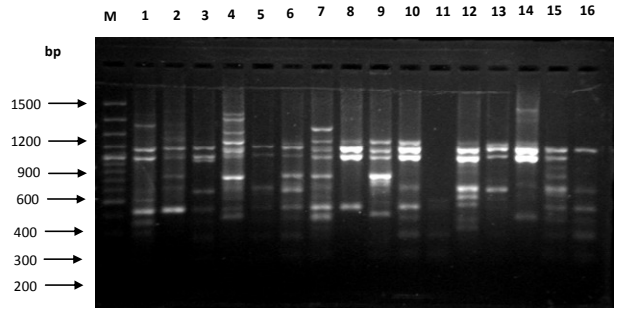

Fig. 5: RAPD marker *OPA-10* showing the banding pattern of the isolates of *M. anisopliae* var. *anisopliae* (1-16 in order), SKCJ2, LSVY, LSVT2, NLWN, LSTC2, CNCG, CNGX, CNHB3, CNHN1, CNGU3, CNZH, CNXJ5, LSNA1, CNXZ , LSHY and CNGD8

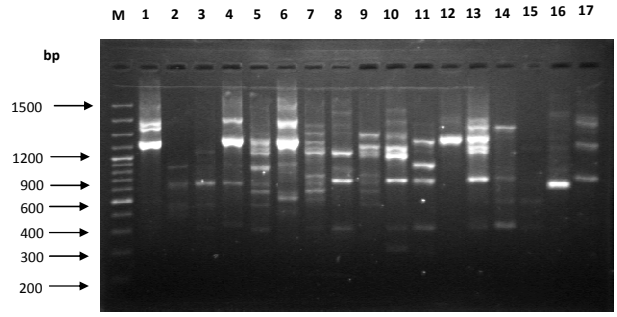

Fig. 6: RAPD marker *OPAB-4* showing the banding pattern of the isolates of *M. anisopliae* var. *anisopliae* (1-17 in order), CNSN2, CNCG, CNGD8, CNHB3, CNHB1, CNHE, CNGD7, CNGD6, CNYN2, CNXJ7, LSNM2, NLHN2, LSPK, CNZH, CNHN2, CNHN3 and CNGD2

Research on genetic differences in insect pathogenic fungi correlated with a certain host has revealed diversity of polymorphisms. Cravanzola *et al.* (1997) recorded maximum genetic similarity among *Beauveria brongniartii* from *Melolontha* spp., while, *I. fumosoroseus* strains from white fly, *Bemisia tabaci* were separated into different

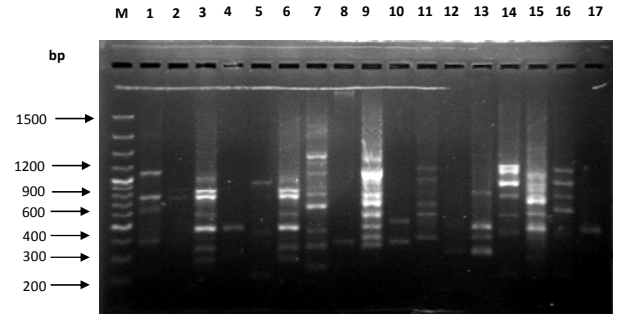

Fig. 7: RAPD marker *OPAB-09* showing the banding pattern of the isolates of *M. anisopliae* var. *anisopliae* (1-17 in order), CNGD2, CNZH, CNGD6, CNGD4, CNHB3, CNHN3, CNGD5, CNXZ, CNYN4, CNXJ1, LSNA2, NLWN, LSHY CNXJ6, CNHN4, CNGD9 and CNGD1

groups (Tigano-Milani *et al.,* 1995) and the level of genetic distinction varies with the quantity of samples, collection approaches (McDonald and Martinez, 1990; Fegan *et al.,* 1993).

By using ten RAPD primers, 62 isolates of *M. anisopliae* from diverse soil ecosystems, 60 distinct phenotypes were observed. This showed the amount of variation that may be recorded within and among *M. anisopliae* populations. The cluster analysis of the isolates of *M. anisopliae* by RAPD showed genetic variability among different isolates and at least three primers were able to distinguish different entomopathogenic fungi, the results somewhat agree with that of the previous effort which reports that RAPD markers were capable to locate a large quantity of genetic differentiation between the strains of *Entomophaga grylii* and *M. anisopliae* (Bidochka *et al.,* 1995: Velásquez *et al.,* 2007). The current findings are in accordance with that of Cobb and Clarkson (1992), in which the RAPD-PCR of *M. anisopliae* var. *anisopliae* showed considerable level of polymorphism within the same group and no association of the genotype was observed between the original insect hosts. Castrillo *et al.* (1999) observed minimal genetic differentiation among the pathogen's population, as in case of *B. bassiana* above 89% similarity was recorded and few strains showed very little variability that couldn't separate few strains from some populations presenting larger resemblance to the isolates from other gathering locations.

Similar studies were done by Bidochka *et al.* (2000), their research showed that the isolates of *M. anisopliae* characterized by RAPD and VCG are not always homologous, it suggests that genetically similar fungi, based on RAPD banding pattern and vegetative compatibility, were more likely to harbor genetically related dsRNA, and that dsRNA elements in *M. anisopliae* are horizontally transferred to genetically related isolates or are maintained through clonal lineages.

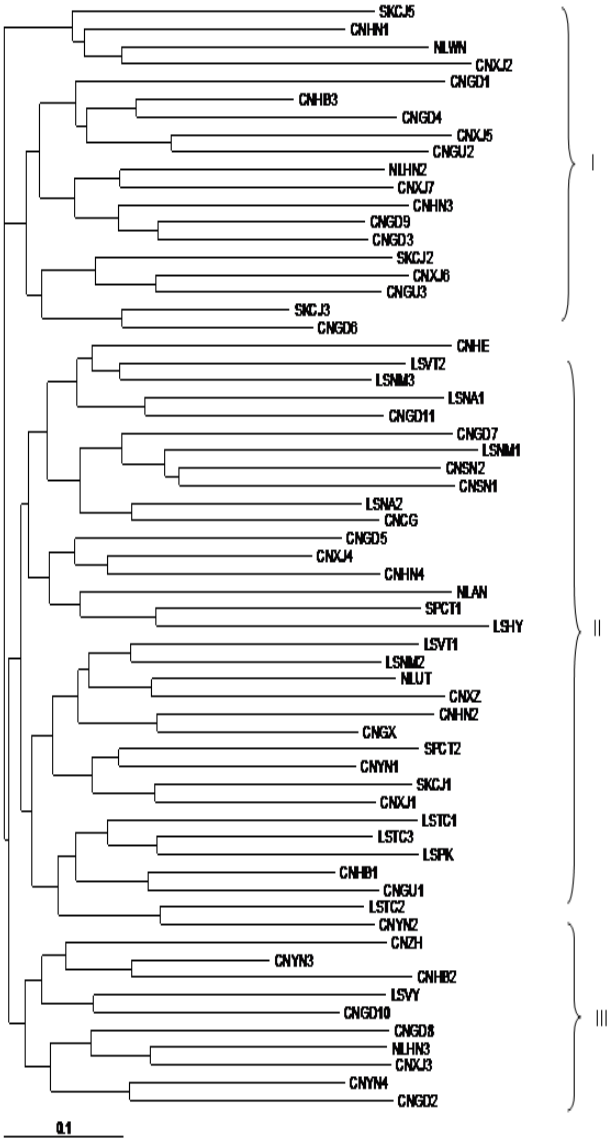

Fig. 8: Dendogram based on RAPD analysis of 62 isolates of *M. anisopliae* var. *anisopliae* from different geographical origins. (CN= China, GD=Guangdong, CG= Chongqing, HB= Hubei, ZH= Zhejiang, HN=Hainan, YN= Yunan, HE= Henan, GX= Guangxi, GU= Guizhou, XZ = Xizang (Tibet) and XJ= Xinjiang), (LS= Laos, NA= Namphao, VY= Viengxay, VT= Vientiane, NM= Namsanam, TC= Tadchampa, PK= Khouay Prefecture and HY= Houayin), (SK= South Korea, CJ= Cheju), (NL= Netherlands, HN= Heteren, WN= Wageningen, UT= Utrecht and AN= Arnhem) and (SP= Singapore, CT= City)

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