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Genetic Diversity of an Alien Invasive Plant Mexican Sunflower (*Tithonia diversifolia*) in China

Jing Yang, Ling Tang, Ya-Li Guan, and Wei-Bang Sun*

Mexican sunflower is a native species of North and Central America that was introduced into China early last century, but it has widely naturalized and become a harmful invasive plant in tropical and subtropical regions in South China. Inter-simple sequence repeat (ISSR) markers were employed to assess genetic diversity and variation in Mexican sunflower populations from China and neighboring regions. The karyotypes of populations were also studied. Our research showed high levels of genetic diversity in all populations. The lowest genetic diversity estimates were represented in two populations in Laos, suggesting prevention of new introductions into Laos is critical. Partitioning of genetic variance revealed that genetic variation was mostly found within populations, and unweighted pair group method with arithmetic means (UPGMA) analysis showed that the introductions into China and Laos were independent. There were no obvious correlations between genetic relationships and geographic distance of populations in China, consistent with the human associated dispersal history of Mexican sunflower. Previous cytological data and our chromosome count (2n = 34) and karyotype analysis showed chromosome stability among populations. The high levels of genetic diversity within invasive Mexican sunflower populations could be challenging for its management in China, and further expansion and potential negative effects on ecological systems of this plant should be monitored.

Nomenclature: Mexican sunflower, *Tithonia diversifolia* (Hemsl.) A. Gray.

Key words: High genetic diversity, ISSR, constant karyotype, cluster analysis, tree marigold.

Invasions by alien species are serious threats to both natural and managed ecosystems worldwide (Mack et al. 2000). Identifying future invaders and taking effective steps to prevent their dispersal and establishment constitutes an enormous challenge to both conservation and international commerce. Mexican sunflower, native to North and Central America, has been widely introduced to Asia, Africa, America, and Australia for ornamental use, green manure, and erosion control, but now has been reported to be naturalized and aggressively invading in Southeast Asia, South Africa, and the Pacific region (Henderson 2001; Lazarides et al. 1997; Meyer 2000; Varnham 2006; Xu et al. 2007). An investigation in Nigeria reported some farmers have abandoned their lands due to the difficulty of controlling Mexican sunflower by hand weeding and hoeing (Chukwuka et al. 2007).

In Yunnan Province (390,000 km^2), our group led an investigation in 2004 (Wang et al. 2004), and recorded the distribution of Mexican sunflower in nine counties and 53 towns (47% of Yunnan's total territory). Plant communities with Mexican sunflower as the dominant species have been found by our field investigations in other provinces of China, including Guangdong, Guangxi, Fujian, Hainan, Hong Kong, and Taiwan. In the community structure survey (Wang et al. 2004), six populations in Yunnan Province were sampled and all the cover grades (according to Braun-Blanquet cover-abundance scale) of Mexican sunflower reached a maximum degree of five (75 to 100% cover range), and its aboveground biomass fresh weights ranged from 22.4 kg to 31.6 kg m^{-2}, which was significantly higher than 0.79 kg to 1.87 kg m^{-2} of its companion species. Field observations revealed that Mexican sunflower can adapt to multiple habitats such as roadsides, river banks, disturbed or abandoned sites, and sun-exposed ecosystems, and can invade fields around farmlands, nursery gardens, and banana orchards.

Mexican sunflower grows as a shrub-like perennial in Yunnan Province, but it is an annual in its native regions. According to investigations by Wang et al. (2004), flowering begins in October and in a typical mature population, 80,000 to 160,000 seeds m^{-2} can be produced annually, 70% of which were fully developed. Germination rates at 25 C ranged from 18 to 56%. The thousand-seed weight ranged from 4.58 g to 6.53 g. The pubescent seed with a pappus can be dispersed by wind, and be carried over large areas by vectors such as humans, livestock, and water currents. Field observations (Wang et al. 2004) indicated that clonal proliferation was common, especially during the rainy reason. Adventitious roots and young shoots emerge from nodes on the lower or prostrate branches, and clonal growth results, contributing to extensive horizontal expansion of patches. It could be inferred that Mexican sunflower might first develop from limited seed dispersal in the new range, and then small populations could gradually establish by both sexual and clonal reproduction (Wang et al. 2004, 2008). Experiments by Tongma et al. (1998, 1999) on allelopathic effects of Mexican sunflower revealed decreases in shoot and root growth of test plant species when grown in soil previously planted with Mexican sunflower or soil treated by water leachates of its leaves, but there was no allelopathic influence on test plant seed germination. Differing allelopathic effects (including both inhibitory and stimulatory) were observed on seedling growth of other test plants (Oyerinde et al. 2009). Allelopathy might partly explain why in sampled Mexican sunflower populations, species diversity and the abundance of companion plants were both low (Wang et al. 2004).

Initial establishment and subsequent range expansion of founding populations result in reduced fitness when selection pressures are generated by the novel environment. On the basis of theories first proposed by Fisher (1930), if successful invasion requires adaptation in response to selection, the rate of spread into new environments will depend on the amount

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* First, second, and fourth authors: Research Assistant, Graduate Student, and Professor, Kunming Botanical Garden, Kunming Institute of Botany, The Chinese Academy of Sciences, 132 Lanhei Road, Heilongtan, 650204, Kunming, Yunnan, China; third author: Associate Professor, Biotechnology Department, College of Life Science, Hainan Normal University, Haikou, Hainan, China. Jing Yang and Ling Tang contributed equally to the work and are joint first authors. Corresponding author's E-mail: wbsun@mail.kib.ac.cn

Table 1. Locations and genetic diversity indices of 16 Mexican sunflower populations sampled for ISSR research.

Population and location	Sample size	Longitude E	Latitude N	Percentage of polymorphic loci (P)	Nei's gene diversity (*H*)	Shannon's diversity index (*I*)
NL (Ninglang)	22	27°21′–26°58′	100°26′–100°31′	70.77	0.3033(0.2073)	0.4369(0.2919)
KM (Kunming)	18	24°55′	102°38′	81.54	0.3149(0.1807)	0.4634(0.2499)
BN (Banna)	23	21°55′–22°30	100°55′–102°3′	81.54	0.3293(0.1800)	0.4802(0.2510)
LC (Lincang)	19	24°4′	99°45′	73.85	0.3019(0.2023)	0.4378(0.2837)
PE (Puer)	20	22°45′	100°57′	81.54	0.3254(0.1752)	0.4767(0.2458)
YJ (Yuanjiang)	22	23°37′	101°56′	73.85	0.2993(0.1978)	0.4360(0.2784)
MIL (Mile)	21	24°24′	103°26′	72.31	0.2842(0.1957)	0.4175(0.2768)
ML (Menglian)	21	24°36′	98°48′	81.54	0.2978(0.1753)	0.4441(0.2434)
XP (Xinping)	22	24°5′	101°58′	80.00	0.3219(0.1843)	0.4698(0.2571)
RL (Ruili)	18	24°9′–28°52′	97°40′–98°22′	78.46	0.3006(0.1934)	0.4411(0.2680)
HN (Hainan)	18	19°0′–19°13′	109°25′–109°52′	76.92	0.3083(0.1890)	0.4507(0.2662)
NN (Nanning)	21	22°46′	108°24′	76.92	0.3087(0.1885)	0.4514(0.2651)
ZJ (Zhanjiang)	22	20°27′	110°07′	81.54	0.3024(0.1817)	0.4482(0.2502)
XM (Xiamen)	20	24°26′	118°05′	78.46	0.3003(0.1857)	0.4426(0.2604)
PAKSONG	20	15°11′	106°13′	60.00	0.1937(0.1920)	0.2950(0.2749)
KHOUANG	22	19°16′	103°23′	58.46	0.2065(0.1958)	0.3103(0.2831)
Average				75.48	0.2937(0.1890)	0.4314(0.2654)
Taxa				84.62	0.3626(0.1689)	0.5226(0.2356)

of additive genetic variation present in the invading population. Typically, in newly established populations, only a small fraction of the available genetic variation from native gene pools was introduced; thus, genetic drift during colonization might bring about reduced genetic variation (Sakai et al. 2001). In recent molecular genetic studies of invasion routes, multiple introductions were a common source of greater levels of genetic diversity (Facon et al. 2003; Gaudeul et al. 2011; Kang et al. 2007; Kolbe et al. 2004) and an explanation for the ''paradox of invasion biology'' whereby invasive populations overcome low variability associated with founder effects and adapt to new environments (Dlugosch and Parker 2008; Handley et al. 2011; Roman and Darling 2007). Also, different colonizing populations are likely to be genetically divergent with different levels of genetic variation (Sakai et al. 2001). Gene flow between populations could result in the spread of invasive genotypes or alternatively prevent evolution of invasiveness by ''swamping out'' locally beneficial alleles (Kirkpatrick and Barton 1997), and could augment diversity over the long term (Dlugosch and Parker 2008). Population genetic analysis based on variable molecular markers can provide information about the routes, multiple introductions, source populations, and expansion mechanisms of invasive species in new territories (Dodet et al. 2008; Geng et al. 2007; Grimsby et al. 2007; Schaal et al. 2003). Such information is essential for effective management of existing populations and for developing general principles to predict and prevent the occurrence of new invasions (Abdelkrim et al. 2007; Ward et al. 2008a).

We have previously studied growth, reproduction and community characteristics of Mexican sunflower and its invasive biological characteristics in China (Wang et al. 2004, 2008). In this study, genetic diversity was measured with inter-simple sequence repeat (ISSR) markers in order to understand regional patterns of genetic variation and genetic structure. Using primers that amplify sequences between two simple sequence repeat loci, ISSR markers sample multiple loci throughout the genome simultaneously, yielding a highly variable marker system useful for fingerprinting and diversity analysis (Tang et al. 2009). Because this marker system requires no previous sequence information and provides good estimates of genetic diversity (Ward 2006), ISSR markers have been widely used in analyzing invasive plant population genetics. For example, ISSR analysis in Canada thistle [*Cirsium arvense* (L.) Scop.] (Slotta et al. 2006), suggested that multiple introductions and continued gene flow between populations was one cause of its continued success in North America; in ragweed parthenium (*Parthenium hysterophorus* L.) (Tang et al. 2009), a new introduced population was discovered based on its genetic differentiation and higher levels of genetic diversity; and in yellow toadflax (*Linaria vulgaris* P. Mill.) (Ward et al. 2008b), ISSR markers showed that multiple introductions have occurred, followed by extensive genetic recombination.

Karyotype changes discovered in invasive *Carpobrotus* spp. (Verlaque et al. 2011) and johnsongrass [*Sorghum halepense* (L.) Pers.] (Cai et al. 2006) have revealed hybridizations of invasive species with nonnative and native species. To determine if there are karyotype variations in Chinese Mexican sunflower populations compared with the karyotype of populations in its native habitat, and to provide information on genome structure characteristics for future research, we also carried out cytological studies on 13 populations.

Materials and Methods

Sampling Methods and DNA Extraction. From November 2007 to 2008, 16 populations of Mexican sunflower were sampled for ISSR research (Table 1) and 13 were sampled for chromosome studies (Table 2). In the 16 populations for genetic diversity investigation, 14 were from four provinces of China and two were from Laos (Figure 1).

For each population, leaf material from 15 to 25 individuals was collected at intervals of at least 20 m. Plant material was dehydrated in sealed plastic ziplock bags containing silica gel. Total genomic DNA was extracted from dry leaves according to the modified CTAB method (Doyle 1991). DNA quality and quantity were determined visually under ultraviolet light on 1% agarose gels.

ISSR–PCR (Polymerase Chain Reaction) Amplification. One hundred ISSR primer pairs designed at the University of British Columbia, Canada, were screened for polymorphisms in eight Mexican sunflower samples from different populations. Ten primer pairs that produced clear and reproducible

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Table 2. Localities, altitudes, karyotype formulas (2n), KA (karyotype asymmetry), and voucher numbers of 13 Mexican sunflower populations in China sampled for karyotype analysis. Voucher (KUN) indicates collector and collector number of voucher specimen deposited in Herbarium of Kunming Institute of Botany (KUN), the Chinese Academy of Sciences, Kunming, Yunnan, China.

Population	Altitude	Karyotype formula (2n)	Karyotype asymmetry	Voucher (KUN)
YJ (Yuanjiang)	750m	26m + 8sm	2A	Y. Zhou 201
ML (Mengla)	550m	26m + 8sm	2A	S. H. Wang 037
HK (Hekou)	76m	28m + 6sm	2A	G. Chen 031
BC (Binchuan)	1,250m	30m + 4sm	2A	G. Chen 092
PE (Puer)	1,350m	30m + 4sm	1A	Y. Zhou 089
NGH (Nangunhe)	1,150m	28m + 6sm	1A	Y. Zhou 073
LC (Lincang)	950m	34m	1A	S. H. Wang 016
FN (Funing)	1,100m	30m + 4sm	1A	G. Chen 043
MZ (Mengzi)	1,550m	24m + 10sm	2A	G. Chen 024
JH (Jinghong)	650m	34m	1A	Y. Zhou 126
KM (Kunming)	1,823m	34m	1A	S. H. Wang 053
XM (Xiamen)	117m	18m + 16sm	2A	L. Tang 015
HN (Hainan)	30m	22m + 12sm	2A	S. H. Wang 019

fragments were selected for further analysis (Table 3). ISSR–PCR amplifications were conducted in a total volume of 15 µl per sample, which contained 0.9 U of Taq polymerase (Takara, Co., Dalian, China), 1× PCR buffer (with 1.5 mM Mg^{2+}), 0.25 mM dNTP each, 0.6 µM of each primer, and 50 ng template DNA. PCR was performed (PTC-100 thermal cycler, MJ Research, Inc., Cambridge, MA) using a program of one cycle of 97 C for 4 min, followed by 40 cycles of 94 C for 1 min, 50 to 52 C for 1 min, 72 C for 1.5 min, with a final elongation at 72 C for 10 min. Six µl of each amplification product were resolved electrophoretically on 1.6% agarose gel buffered with 1× TBE, stained with ethidium bromide, and digitally photographed under ultraviolet light.

ISSR Data Analysis. ISSR fragments were scored for presence (1) or absence (0) and, based on the ISSR phenotypes, a distance matrix was constructed. The following parameters were calculated using software POPGENE version 1.31 (Yeh et al. 1997), assuming Hardy–Weinberg equilibrium: (i) the percentage of polymorphic loci (P); (ii) Nei's genetic diversity (*H*), (Nei 1987); (iii) Shannon's index of diversity (*I*), (Lewontin 1972); (iv) coefficient of gene differentiation (*Gst*); and (v) gene flow (*N*m) (Wright 1931). Analysis of molecular variation (AMOVA) was used to analyze the hierarchical genetic structure using Arlequin version 3.11 (Excoffier et al. 2005), by which the partitioning of genetic diversity within and among populations was tested. Nei's genetic distance and genetic identity between all pairs of populations were also calculated, and a dendrogram was generated from the distance values using UPGMA cluster analysis based on Nei and Li similarity coefficients (Nei and Li 1979), both using POPGENE 1.31. Bootstrap analysis with 1,000 replicates was calculated by Populations 1.2.30 (http://bioinformatics.org/~tryphon/populations/).

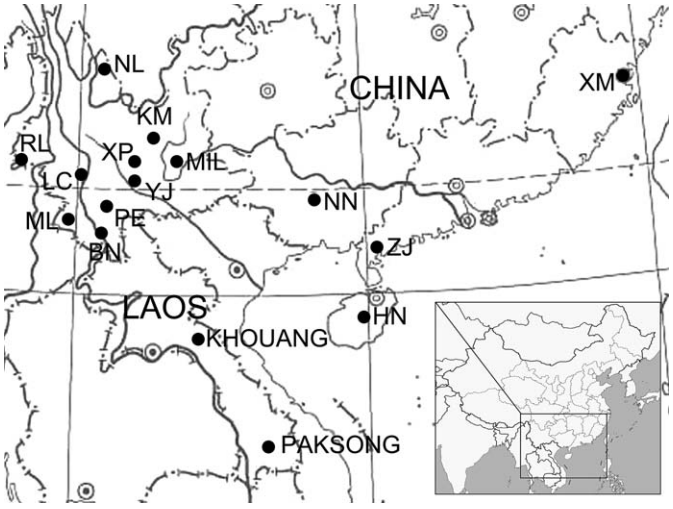

Figure 1. The geographic locations of Mexican sunflower populations sampled for ISSR research. Solid black dots indicate the locations and other dots represent capital cities.

Karyotype Analysis. Seedlings germinated from the seeds of the 13 Mexican sunflower populations (Table 2) were planted in Kunming Botanical Garden (KBG). For cytological observations, vigorously growing root tips were pretreated in 0.002 mol L^{-1} 8-hydroxyquinoline solution at 25 C for 120 min, then fixed with Carnoy's fluid (absolute ethanol : glacial acetic acid 3 : 1 by volume) at 4 C for at least 30 min. The fixed roots were hydrolyzed in a 50 : 50 mixture of 1 N HCl and 45% acetic acid at 60 C for 1 min, stained with 1% aceto-orcein for 1 h, and squashed for cytological observation. Slides were made permanent using the standard liquid nitrogen method.

For each population, karyotypes of somatic chromosomes at metaphase were determined by at least 10 well-spread metaphases, all from three or more plants. Descriptions of the positions of the centromeres on metaphase chromosomes were as specified by Levan et al. (1964). Karyotype asymmetry was estimated according to Stebbins (1971).

Results and Discussion

Karyotypes. In mitotic metaphase cells of Mexican sunflower, all 13 populations showed a stable chromosome count of 2n = 34, x = 17. No polyploid individuals were found. Karyotype formulas are listed in Table 2. Of the 34 chromosomes, metacentric chromosomes varied from 18 to 34, submetacentric chromosomes from 0 to 16. There were no telocentric or subtelocentric chromosomes observed in any of the individuals examined. Karyotype asymmetry was categorized as Type 1A or 2A, which infers a low level of asymmetry in the whole set of chromosomes.

In the genus *Tithonia*, a chromosome count of 2n = 34 and x = 17 is dominant, and polyploidy has not been found (Goldblatt and Johnson 2012). Our study further confirmed this count. Among the 13 populations studied, karyotype

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Table 3. The effective primers and their sequences used in the inter-simple sequence repeat (ISSR) analysis.

Primer Code	Primer sequence (5′–3′)	Annealing temperature (C)	No. of loci	No. of polymorphic loci	Polymorphism (%)
809	(AG)8G	53	9	6	77.78
812	(GA)8A	50	8	7	87.5
827	(AC)8G	52	4	4	100.00
834	(AG)8YT	51	9	8	88.89
835	(AG)8C	50	5	4	80.00
836	(AT)8YA	49	6	5	85.71
840	(GA)8YT	50	5	4	80.00
847	(CA)8RC	51	9	9	100.00
855	(AC)8YT	52.5	4	3	80.00
891	HVH(TG)7	50	6	5	83.33
Total			65	55	84.62

characteristics did not appear to differ. In a study by Xie and Zheng (2003), a karyotype formula of 2n = 34 = 26m + 8sm was found in a population from Hainan, (HN in our study), yet we observed 2n = 22m + 12sm in HN. A study by Wang and Li (1987) found 2n = 24m (4SAT) + 10sm (2SAT), but gave no details about the distribution. Alcorces de Guerra et al. (2007) examined material from Venezuela, and the formula was n = 16m + 1sm. The small deviations in these data might be caused by different pretreatment methods and/or microscopic conditions, and are not sufficient proof of chromosome variation. The 1A or 2A karyotype asymmetry and the absence of telocentric or subtelocentric chromosomes revealed stability in Mexican sunflower's chromosome sets, indicating that little translocation has taken place in its evolution. Although no obvious karyotype changes were discovered between native and invasive populations of Mexican sunflower, cross-species hybridization might yield unpredictable outcomes. Our results provide information on current genome structure characteristics for future investigations in this field.

Genetic Diversity and Variation. Ten ISSR primer pairs generated 65 scorable bands across the 329 individual samples from 16 populations, 55 of which were polymorphic (84.62%) at the species level (Table 3). Genetic diversity estimates are given in Table 1. For the 16 populations, the percentage of polymorphic loci (*P*) ranged from 58.46 to 81.54%, with a mean of 75.48%. Mean values of Nei's genetic diversity (*H*) and Shannon index of diversity (*I*) were 0.2937 and 0.4314, respectively. The BN population (China) showed the highest genetic diversity (P = 81.54%, *H* = 0.3293, *I* = 0.4802), and the PAKSONG population (Laos) presented the lowest (P = 60.00%, *H* = 0.1937, *I* = 0.2950). Both populations from Laos (PAKSONG and KHOUANG) showed lower diversity compared with the 14 populations from China.

Using the program POPGENE, the total gene diversity of these 16 populations was *Ht* = 0.3604, the within population genetic diversity was *Hs* = 0.2939, and the coefficient of gene differentiation *Gst* = 0.1845. AMOVA using Arlequin revealed that 11.90% of genetic variation was distributed among populations within groups, and 81.59% was within populations (Table 4). Based on geographic distribution, the 16 populations were divided into six groups: Yunnan (KM, NL, BN, LC, XP, MIL, ML, RL, YJ, PE), Hainan (HN), Guangxi (NN), Guangdong (ZJ), Fujian (XM), and Laos (PAKSONG, KHOUANG). A total of 6.51% genetic variation existed among the six groups, suggesting a low level of geographic differentiation. Both of the methods used to analyze our data suggested that the genetic variation occurred mostly within populations.

Compared with data of ISSR analysis on invasive plants (Gutierrez-Ozuna et al. 2009; Li et al. 2007; Ren et al. 2010; Ward et al. 2008b), our research showed that Mexican sunflower has a very high genetic diversity. In the introduction history of Mexican sunflower (Wang et al. 2004), after the first record in Banna (BN) in 1936 as ornamental plants, more specimen records indicated that in the 1950s in Banna, Luxi, Gengma and Cangyuan counties (all in Yunnan Province), Mexican sunflower was planted in villages and along the roadsides for landscaping. In the 1970s, farmers grew more Mexican sunflower as green manure and then abandoned it because of the emergence of chemical fertilizers. The high levels of genetic diversity might reflect multiple introductions or genotypic diversity from the original regions. Although the breeding system of Mexican sunflower has not been extensively studied, the high within-population genetic diversity and lower levels of genetic diversity among populations implies outcrossing in this species (Hamrick and Godt 1996).

In the 14 populations in China, BN showed the highest genetic diversity, and from this location the first specimen record of Mexican sunflower was collected in 1936 (Wang et al. 2004). Having been introduced a relatively long time ago, the high diversity could be explained by the long-term progression of evolution. The two populations from Laos (PAKSONG, KHOUANG) showed relatively low genetic diversity compared with Chinese populations, suggesting that the original introductions of Mexican sunflower into Laos were less genetic diverse and independent from those in China. This finding indicates that in Laos, preventing introductions of new genotypes might be helpful to maintain

Table 4. The analysis of molecular variance (AMOVA) for 16 Mexican sunflower populations in China.

Source of variation	Degrees of freedom	Sum of squares	Variance component[a]	Variation (%)	P value
Among groups	5	331.431	0.75519a	6.51	0.0108
Among populations/groups	10	379.209	1.38006b	11.90	< 0.001
Within populations	313	2961.621	9.46205c	81.59	< 0.001

[a] Different letters following value indicate significant difference at P≤0.05.

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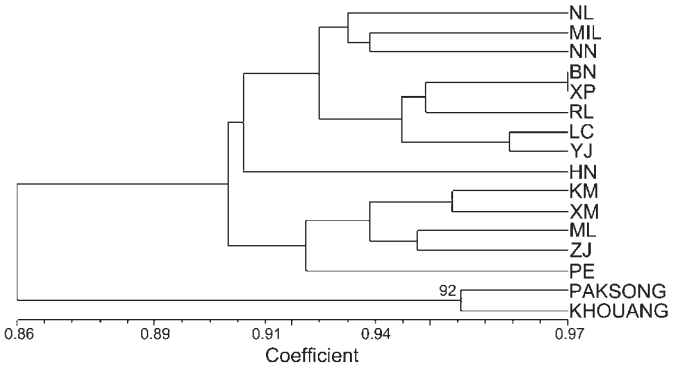

Figure 2. Dendrogram of unweighted pair group method in arithmetic means (UPGMA) cluster analysis among 16 Mexican sunflower populations based on Nei's genetic identity. Bootstrap values above 90% are shown.

a lower level of genetic diversity and reduce the capacity to resist control measures. A higher degree of genetic diversity can confer higher fitness; examples show that more diverse weed populations have more resistance to diseases, pests, and herbicides (Excoffier et al. 1992; O'Hanlon et al. 2000). Although neutral markers were used in this study and adaptive variability, rather than neutral variability, controls the adaptability of individuals of populations, neutral and adaptive variability are often considered to be correlated, although not always (Milligan et al. 1994). Thus, the potential invasive expansion of Mexican sunflower should not be underestimated.

Genetic Structure and Population Relationships. Estimates of Nei's genetic distance and genetic identity between all pairs of populations ranged from 0.0301 to 0.2029 and 0.8164 to 0.9703 (data not shown). Figure 2 shows the UPGMA cluster dendrogram. The two Laos populations (PAKSONG and KHAUANG) were separated from the clusters of Chinese populations with high bootstrap support (92%), which suggests that the introductions of Mexican sunflower into them were independent. The dendrogram showed no obvious correlations between genetic distance and geographic distance of the populations in China. Mantel tests were conducted using tools for population genetic analyses (TFPGA) software (www.marksgeneticsoftware.net/tfpga.htm), and the probabilities of the observed correlations were estimated using 10,000 random permutations of matrix elements. A coefficient of $r^2 = 0.1592$ ($P = 0.1490 > 0.05$) was obtained, suggesting no significant correlation between them. Based on introduction history of Mexican sunflower in China, human-mediated long-distance transport of seeds might be responsible for the observation that geographically distant populations appear to share genotypes.

The total genetic diversity for Mexican sunflower was $Ht = 0.3604$, and the mean hererozygosity within populations was $Hs = 0.2939$, which indicated that the genetic variation mostly occurred within populations. The coefficient of gene differentiation was $Gst = 0.1845$. According to Wright (1984), $Gst > 0.25$ indicates high differentiation among populations; genetic differentiation among populations of Mexican sunflower was not high. Based on Wright (1984), gene flow $Nm = 2.2071$ was high and could have prevented population genetic differentiation (Franklin 1980). This could be correlated with multiple seed dispersal mechanisms.

Our earlier studies investigated the rapid propagation rate and other biological traits of Mexican sunflower in China (Wang et al. 2004, 2008), and this research presents genetic data. The high level of diversity within populations could allow adaptation to occur, potentially reducing the effectiveness of control methods. In addition, the ornamental value of this plant might attract further introductions and increase the risk of invasion, which has not been widely publicized (Dawson et al. 2008). Our research clarifies the invasive status of Mexican sunflower in China; thus, caution should be exercised in other areas of introduction.

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