INTRODUCTION

Geographic isolation due to the uplift of mountain chains and climatic fluctuations associated with glacial oscillations can cause great variations in both the morphology and geographic distribution of many species (Liu et al., 2013). Genetic differentiation in local species may be the result of mutation or genetic drift, while gene flow may adapt to the native conditions to either restrict evolution or accelerate evolution by spreading new genes (Montgomery, 1987). The adaptation of species to a specific geographic habitat is partly determined by a series of historical events. In most cases, a species will extend the range of its habitat until barriers prevent dispersal, where the obstacles are mostly large and conspicuous, such as high mountains, wide oceans, deserts, or other geographic features, which form a relatively isolated...
network region (He & Jiang, 2014). Studies have also shown that species distribution patterns and structures are also affected by many external ecological factors, including climate, predators, and competitors (Montgomery, 1987).

Southwest China is one of the most important biodiversity hotspots, and it is characterized by extremely complex geographically isolated habitats (He & Jiang, 2014). The altitudes in this region differ greatly where they range from 300 m in Nujiang valley to Mount Gongga at more than 7,556 m above sea level (Zhao & Yang, 1997). Most of these parallel mountain chains are oriented in a north-south direction, and they are divided by very deep river canyons. The highly complex terrains may provide a relatively stable model of ecologically diverse habitats and glacial refugia because the vegetation and habitats only shifted vertically by a few hundred meters during the Pleistocene climate fluctuation (He & Jiang, 2014). Thus, this particular geographic environment is a natural cradle that maintains species richness. A well-known biogeographic boundary exists in southwest China known as the Tanaka Line (Tanaka, 1954; Zhu & Yan, 2002). The Tanaka Line is considered to be a straight line between approximately 28°N, 98°E and 18°45′N, and 108°E, which divides the two floristic subkingdoms of East Asia, with the Sino-Japanese to the east and the Sino-Himalayan to the west (Li & Li, 1997). The genetic diversity and population subdivisions are markedly different on either side of the Tanaka Line (Fan et al., 2013; Tian et al., 2015), which makes this an ideal region to study the effects of different factors on species diversification and evolution.

Bombax ceiba Linn. (Malvaceae), known as the red silk cotton tree, is a tall, drought-tolerant, and arbor tree species with a wide distribution on both sides of the Tanaka Line (Chaudhary & Khadabadi, 2012). Natural populations of this tree species are widespread in South-East Asian countries at altitudes below 1,400–1,700 m (Li, 1984). In China, B. ceiba occurs naturally in subtropical regions, such as the dry-hot valleys of southwest China (Figure 1). The fresh leaf sample was placed onto silica gel and dried immediately. The latitude and longitude were recorded for each sampled population using GPS system (Garmin, Taiwan), and the locations are listed in Appendix 1. Voucher specimens were preserved and archived in the herbarium of Southwest Forestry University, China.

2 | MATERIALS AND METHODS

2.1 | Plant materials

We collected 224 samples from 17 populations of B. ceiba in the dry-hot valleys of southwest China (Figure 1). The fresh leaf sample was placed onto silica gel and dried immediately. The latitude and longitude were recorded for each sampled population using GPS system (Garmin, Taiwan), and the locations are listed in Appendix 1. Voucher specimens were preserved and archived in the herbarium of Southwest Forestry University, China.

2.2 | DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from leaf tissues using DNA Extraction Kits (TIANGEN, Beijing, China) according to the manufacturer’s protocol. The concentration and quality of the DNA were detected using a spectrophotometer. Six highly variable pairs of EST-SSR primers (Appendix 2) were used to detect polymorphisms in B. ceiba. PCR amplification was performed according to the procedure described previously by Ju, Ma, Xin, Zhou, and Tian (2015). All of the high-quality PCR products were sequenced using the amplified forward and reverse primers with an ABI 3730xl Sequence Analyzer (Life Technologies, Carlsbad, CA, USA).

2.3 | Data analysis

The sequences obtained were aligned using MUSCLE (Edgar, 2004) and revised manually in MEGA 7 (Tamura et al., 2011). Hardy–Weinberg equilibrium and linkage disequilibrium were assessed for each population and microsatellite locus pair with PopGen version 32 (Yeh, Yang, & Boyle, 1999). Neutral microsatellite loci were used for the population genetic analyses. Genetic diversity parameters comprising the allele size (A), effective number of alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), and polymorphism information content (PIC) were calculated for each locus using GenAIEx version 6.501 (Peakall & Smouse, 2012) and PIC_CALC version 0.6. Correlation analyses of the genetic similarity and geographic distances among the 17 populations were calculated using PopGen version 32 and based on a Mantel test (Mantel, 1967) with 999 matrix randomizations using GenAIEx version 6.501. According to the genetic distance matrix calculated among the 224 samples with GenAIEx version 6.501, the similarity matrix was subjected to cluster analysis using the UPGMA algorithm with NTSYS-PC version 2.0 and a dendrogram was generated (Rohlf, 2000). Interpopulation and intrapopulation genetic differentiation were partitioned by analysis of molecular variance (AMOVA)
using ARLEQUIN version 3.5.2.1 (Excoffier & Lischer, 2010) with 1,000 random permutation tests. The population genetic structure was determined with the Bayesian clustering approach implemented in STRUCTURE version 2.3.1 (Evanno, Regnaut, & Goudet, 2005). An admixture ancestry model was applied, and 10 independent runs were conducted for each $K$ (1–9) with 50,000 burn-in and 100,000 Markov Chain Monte Carlo iterations. A suitable number of clusters ($K$) were selected as the largest rate of change in the log probability of data between successive $K$ values (Pritchard, Stephens, & Donnelly, 2000), as implemented in STRUCTURE HARVESTER (available online at: http://taylor0.biology.ucla.edu/structureHarvester/). Admixture proportions obtained from replicate simulations at the optimal $K$ were averaged using CLUMPP version 1.1.2 (Jakobsson & Rosenberg, 2007). We then employed MIGRATE-N v3.6 (Beerli, 2006) to explore the direction of historical gene flow among the 17 populations based on the Bayesian clustering results. The geographic locations of genetic discontinuities among populations were determined with BARRIER version 2.2 (Manni, Guerard, & Heyer, 2004).

### RESULTS

#### 3.1 Genetic diversity

In total, 27 alleles were identified in the six SSR loci among the 224 individuals from 17 $B. $ceiba populations. All of the loci conformed to Hardy–Weinberg equilibrium, and they were polymorphic among populations. The number of alleles ($A$) ranged from two to seven (Table 1), and the mean number of alleles was 4.5. $H_0$ and $H_e$ varied from 0.2217 to 0.4486 (mean value = 0.3620) and 0.2424 to 0.6085 (mean value = 0.4622), respectively (Table 1). The PIC value for each locus ranged from 0.2311 to 0.5534, with an average of 0.3874 (Table 1).

The population genetic diversity results obtained at the population level are listed in Table 2, which show that there were clear differences in the numbers of polymorphic bands. The percentage of polymorphic loci per population varied from 66.67% to 100%. Nei’s gene diversity ($H_e$) and Shannon’s index ($I$) ranged from 0.2377 to 0.4775 and 0.3997 to 0.7848, respectively (Table 2).
Cluster analysis showed that all of the *B. ceiba* samples clustered into two groups (cluster 1 and cluster 2). Cluster 1 comprised the BN, YJ, BC, ET, YM, and RH populations. The LC, PE, YD, GM, SD, LJ, LSH, and LL populations were grouped in cluster 2 (Figure 2). The UPGMA dendrogram suggested that the clustering was highly dependent on the geographic origins of populations (Figure 2). The populations northeast of the Tanaka Line belonged to cluster 1, whereas all of the populations southwest of the Tanaka Line belonged to cluster 2. Thus, the populations located on each side of Tanaka Line had relatively different genetic characters. The Mantel test results also indicated that there was a significant correlation between the genetic distances and geographic distances for all of the populations examined ($r^2 = .2553$, $p < .001$, 999 permutations) (Figure 3).

### 3.3 Population genetic structure

According to the six neutral polymorphic markers employed in the population genetic structure analyses using *K* values ranging from 1 to 10, the STRUCTURE simulation obtained the highest peak at *K* = 2 (Figure 4). The two *B. ceiba* subpopulations at *K* = 2 were attributed to those in the southwest and northeast regions with respect to the Tanaka Line. The membership results inferred that the
genetic structure of *B. ceiba* matched with the geographic distribution (Figure 4). The estimation of gene flow suggested that gene exchange existed among the populations (*Nm* = 1.1792; Table 4).

We defined two clusters based on the STRUCTURE results in order to evaluate the direction of historical gene flow among the 17 populations. The gene pool in cluster 1 was mostly attributed to the northeast group and that in cluster 2 was attributed to the southwest group. We performed maximum likelihood analyses with MIGRATE-N using 10 short chains (5,000 trees) and three long chains (50,000 trees), where 10,000 trees were discarded as a burn-in. Interesting patterns of historical gene flow were determined between the two groups where these patterns were relatively symmetrical with slight differences (Table 5). However, all of the slightly asymmetrical patterns were related to a population migration direction from the northeast group to the southwest group (*m*$_{12}$ > *m*$_{21}$).

BARRIER analysis suggested that the largest genetic breaks in many cases agreed with mountainous areas and rivers (Figure 5). Thus, the Gaoligong Mountains separated the LSH and LJ populations, Nujiang River isolated the LJ and LL populations, and the Nushan Mountains divided the LL and SD populations. These are the main barriers that have affected the dispersal of *B. ceiba*.

### DISCUSSION

#### 4.1 Genetic variation

Our analysis based on six microsatellite loci indicated that the PIC values ranged from 0.2311 to 0.5534, with a mean value of 0.3874

| TABLE 3 | AMOVA test results for 224 *B. ceiba* individuals in the 17 natural populations |
| Regions      | Source of variation | df | SSD  | Variance component | Percentage variance (%) | Fixation Index |
|--------------|---------------------|----|------|---------------------|-------------------------|----------------|
| Whole        | Among groups        | 1  | 5.817| 0.01798             | 2.69                    | *F*$_{ST}$: 0.08583 |
|              | Among populations   | 15 | 24.212| 0.03930             | 5.89                    | *F*$_{SC}$: 0.06052 |
|              | Within populations  | 431| 262.949| 0.61009             | 91.42                   | *F*$_{CT}$: 0.02695 |
|              | Total               | 447| 292.978| 0.66737             |                         |                |
| Northern region | Among populations  | 8  | 22.865| 0.09829             | 12.07                   | *F*$_{ST}$: 0.12069 |
|              | Within populations  | 189| 135.347| 0.71612             | 87.93                   |                |
| Southern region | Among populations  | 7  | 14.458| 0.04746             | 6.81                    | *F*$_{ST}$: 0.06810 |
|              | Within populations  | 242| 157.162| 0.64943             | 93.19                   |                |
According to Botstein, White, Skolnick, and Davis (1980), all loci are considered to be reasonably informative. The mean values of $H_o$ and $H_e$ were 0.3620 and 0.4622, respectively (Table 1). Thus, the six SSR loci used for B. ceiba in this study exhibited high polymorphism.

Our analysis of the diversity among B. ceiba populations detected a high level of intrapopulation genetic diversity in B. ceiba ($H_e$: 0.2377–0.4775; $I$: 0.3997–0.7848) (Table 2). In general, species with long history characteristic must have an adequate gene pool to provide sufficient diversity to survive and adapt to complex habitats (Booy, Hendriks, Smulders, Van Groenendael, & Vosman, 2000). Hence, it is important for plant species to retain as much genetic variation as possible to enhance its likelihood of recovery (Cruz et al., 2012). The natural B. ceiba populations had high genetic diversity in this study, possibly because this species is a perennial species with a high potential for outcrossing via entomophilous flowers (Aluri, Srungavarapu, & Kone, 2005). Previously, Nybom (2004) showed that perennial, outcrossing, and widely distributed species exhibit higher levels of genetic variability within populations. Furthermore, this high diversity may be the main factor that allowed B. ceiba to adapt to harsh environments and become the dominant species (Li, 1984) in these dry-hot valleys through a long evolutionary process.

**Table 4** Genetic differentiation and gene flow in the 17 B. ceiba populations

| Locus | $F_{IS}$ | $F_{IT}$ | $F_{ST}$ | Nm |
|-------|---------|---------|---------|----|
| BC1   | 0.0465  | 0.2717  | 0.2362  | 0.8086 |
| BC5   | 0.0331  | 0.0951  | 0.0641  | 3.6508 |
| BC9   | 0.1296  | 0.2665  | 0.1573  | 1.3393 |
| BC10  | 0.0660  | 0.1464  | 0.0861  | 2.6535 |
| BC11  | −0.1405 | 0.1553  | 0.2593  | 0.7140 |
| BC12  | 0.0690  | 0.2683  | 0.2140  | 0.9180 |
| Mean  | 0.0439  | 0.2112  | 0.1749  | 1.1792 |

**Figure 4** STRUCTURE clustering analysis results for B. ceiba populations based on their geographic distribution. Colors represent the population’s probability of populations belonging to either of the two clusters, where blue represents cluster 1 and yellow represents cluster 2. The right-hand figure shows the number of clusters (K) determined for the B. ceiba populations analyzed was the highest peak that was at K = 2. Each vertical bar in the histogram represents a population.
4.2 | Population structure

Clustering analysis based on UPGMA and Bayesian methods suggested that the 17 natural B. ceiba populations could be divided into two genetically divergent clusters (Figures 2 and 4) located on either side of the Tanaka Line. This result is similar to that obtained based on chloroplast DNA data in a previous study by Tian et al. (2015). However, in the present study, we detected the gene flow among populations based on SSR markers. These two types of molecular markers differ in terms of genetic diversity and genetic differentiation, and they have been detected in various plants (Kurokawa, Kobayashi, & Ikeda, 2010; Zeinalabedini, Khayamnekoui, Grigorian, Gradziel, & Martinezgomez, 2010). Combining analyses based on nuclear and chloroplast markers can help to elucidate the evolutionary history of species with different inherited patterns (Mariana & Juan, 2016). Thus, in contrast to the chloroplast fragments, the DNA microsatellites could be used to determine contemporary pollen and seed dispersal (Wolfe, Li, & Sharp, 1987). Variation is influenced by the parental heredity and a high level of mutation rate, which reflects the current genetic structure and distribution of genetic variation (Mariana & Juan, 2016).

The B. ceiba flowers are red and cup-shaped with rich nectar, and they could emit a mild fetid smell to attract a wide range of insects and animals, such as bees, birds, bats, and even monkeys (Aluri et al., 2005). While exploring the B. ceiba flowers, animals contact the stigma and stamens so the pollen can adhere to their head and body to facilitate dispersals. Some bees only collect nectar and move between

| TABLE 5 | MIGRATE analysis for B. ceiba using SSR data |
| Parameter | Percentiles | 0.005 | 0.025 | 0.05 | 0.25 | MLE | 0.75 | 0.95 | 0.975 | 0.995 |
|-----------|-------------|-------|-------|-------|-------|-----|-------|-------|-------|-------|
| $m_{12}$  |             | 0.9401 | 1.0096 | 1.0469 | 1.1677 | 1.2573 | 1.3521 | 1.4998 | 1.5540 | 1.6768 |
| $m_{21}$  |             | 0.8679 | 0.9453 | 0.9824 | 1.0986 | 1.1868 | 1.2821 | 1.4339 | 1.4868 | 1.5950 |

$m_{12}$, migration rate from cluster 1 to cluster 2; $m_{21}$, migration rate from cluster 2 to cluster 1.

**FIGURE 5** BARRIER analysis results showing the spatial separation. The BARRIER analysis results were based on microsatellite data (upper right).
conspecific trees nearby, thereby facilitating pollination (Aluri et al., 2005). This foraging behavior is considered to affect cross-pollination, and it might weaken the genetic structure in the natural populations. In addition, Ashoke (1999) found that the highest number of pollen grains generated per flower by B. ceiba was about 8,863,000 and the maximum atmospheric incidence was 156/m² at 10 hr.

In addition, the $F_{ST}$ analysis showed that the proportion of genetic differentiation among populations accounted for about 0.1749 of the total genetic diversity (Table 4). According to Wright (1978), the differentiation among populations is relatively large (0.15–0.25). Clearly, a positive correlation between the genetic and geographic distances was detected among the populations ($r^2 = .2553, p < .001$) (Figure 3), and thus, topography may be one of the most important factors that have led to differentiation. Natural adaptation probably explains the first level of differentiation within the progenitor B. ceiba population, while habitat fragmentation may have been responsible for the second level of hierarchical variation. The isolation between populations is due to physical barriers in the form of complex terrain with mountains and rivers in southwest China. The genetic structure is expected to be congruent with the geographic arrangement of the mountains and river systems. The genetic distance was relatively large even with a close geographic distance, and genetic discontinuities between the two nearby territories were also identified by BARRIER (Figure 5).

BARRIER analysis based on microsatellite data showed that, in recent times, variations in the topography and climate have contributed to the high endemic biodiversity in southwest China (Myers, Mittermeier, Mittermeier, Da, & Kent, 2000). The extremely complex topography of this region provides ecologically diverse habitats in three dimensions. In addition, the river systems in southwest China are extremely complex, such as the Jinsha River, Lancang River, and the Nujiang River and its tributaries. River canyons have been shaped by tectonism to create uplifts on the Qinghai–Tibet Plateau, which may have existed prior to the rivers (Cheng, Liu, Gao, Tang, & Yue, 2001; Clark et al., 2004). These diverse and stable environments are highly favorable for maintaining species richness. In this study, the southwest and northeast regions contained different genetic structures because of their terrain and large geographic barriers, such as the Hengduan Mountains and Lancang River. Between these two regions, the Tanaka Line may also be an important barrier that divides the B. ceiba natural populations into two parts. Indeed, previous studies have demonstrated that the Tanaka Line currently plays a key role in shaping plant dispersal and it is a habitat–heterogeneity boundary in southwest China (Tanaka, 1954; Zhu & Yan, 2002). The heterogeneous environmental conditions on the Tanaka Line have significantly affected the development and evolution of plant species, that is, a genetic diversity study of Sophora davidii found obvious differences in the population structure on both sides of the Tanaka–Kaiyong Line (Fan et al., 2013). Hence, the Tanaka Line may be responsible for maintaining the major southwest and northeast split in the B. ceiba populations associated with an ecological transition. This major form of isolation may hinder the gene exchange via birds but not pollen dispersal. Hence, this pattern may weaken the specific population structure of B. ceiba on either side of the Tanaka Line. Natural adaptation and physical barriers could explain the divergence among the two subpopulations. Overall, our findings support a hypothesis that the Tanaka Line has contributed to the intraspecific divergence pattern in this species, thereby facilitating the protection and exploitation of wild B. ceiba population resources.

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CONFLICT OF INTEREST

None declared.

AUTHOR’S CONTRIBUTIONS

BT contributed to the conception of the study. BT and YF collected the materials. ZHL and MMJ contributed significantly to analysis and manuscript preparation. MMJ performed the data analyses and wrote the manuscript. CZH contributed the reagents/materials/analysis tools. BT, ZHL, and GFZ helped perform the analysis with constructive discussions. All authors contributed critically to the drafts and gave final approval for publication.

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APPENDIX 1  Locality information and numbers of *B. ceiba* sampled

| Population code | Location          | N  | Geographic coordinates |          |          |          |
|-----------------|-------------------|----|------------------------|----------|----------|----------|
|                 |                   |    | Latitude (N)           | Longitude (E) | Altitude (m) |          |
| BN              | Xishuangbanna, Yunnan | 13 | 21°53’                | 100°59’  | 570      |          |
| YJ              | Yuanjiang, Yunnan  | 9  | 23°31’                | 102°03’  | 850      |          |
| BC              | Binchuan, Yunnan   | 17 | 25°49’                | 100°34’  | 1,430    |          |
| LC              | Lancang, Yunnan    | 13 | 22°32’                | 100°18’  | 1,090    |          |
| PE              | Puer, Yunnan       | 12 | 22°45’                | 100°13’  | 1,340    |          |
| YD              | Yongde, Yunnan     | 22 | 24°02’                | 99°22’   | 1,110    |          |
| GM              | Gengma, Yunnan     | 19 | 23°22’                | 99°38’   | 890      |          |
| HH              | Honghe, Yunnan     |  6 | 23°21’                | 102°24’  | 520      |          |
| LJ              | Luijiang, Yunnan   | 34 | 24°53’                | 98°53’   | 660      |          |
| SD              | Shidian, Yunnan    |  6 | 24°28’                | 99°15’   | 1,100    |          |
| LL              | Longling, Yunnan   |  5 | 24°19’                | 99°01’   | 750      |          |
| RH              | Renhe, Sichuan     |  9 | 26°28’                | 101°44’  | 1,110    |          |
| LSH             | Lushui, Yunnan     | 14 | 25°34’                | 98°52’   | 1,060    |          |
| YB              | Yangbi, Yunnan     |  9 | 25°35’                | 100°13’  | 2,100    |          |
| ET              | Ertan, Sichuan     | 13 | 26°48’                | 101°47’  | 1,100    |          |
| YM              | Yuanmou, Yunnan    | 12 | 25°36’                | 101°49’  | 1,120    |          |
| YY              | Yuanyang, Yunnan   | 11 | 22°57’                | 102°42’  | 600      |          |

N, number of individuals.

APPENDIX 2  Characteristics of nuclear microsatellites used to analyze *B. ceiba*

| Locus | Primer sequence (5’→3’) | Repeat motif | Allele size range (bp) | Ta (°C) | Fluorescent dye | BLAST to hit description [organism] |
|-------|--------------------------|--------------|------------------------|---------|----------------|-----------------------------------|
| BC1   | F: TACTCCGAAAACCTACGGCCTT R: AAAGACGTATCGGTGAAGCG | (CTT)$_7$ | 270–273 | 59 | 6-FAM | Nonintrinsic ABC protein 6, putative isoform 2 [Theobroma cacao] |
| BC5   | F: ACAAAAATGGCGTCTTCTGGG R: GCAGGAGATCCATGGTGATT | (CAG)$_5$ | 128–134 | 60 | 6-FAM | -- |
| BC9   | F: TTTGAAAAAGGGAGGTGTTTGG R: GAGGAGAAGTTATGTGTGGG | (GACT)$_6$ | 134–138 | 57 | HEX | -- |
| BC10  | F: ACCTCCTGCACAGACCATTC R: CATGGGGAATTTTTGTGG | (ACA)$_6$ | 213–216 | 60 | 6-FAM | -- |
| BC11  | F: TGGGAGCTGAGATTTGTACCC R: CCCACTCGATTTTGGATT | (CAGC)$_6$ | 316–320 | 60 | NED | -- |
| BC12  | F: TCCATCCAAATTCCCAGCACA R: GGTTTTCGTGCAAGGAGTCG | (CAG)$_6$ | 147–150 | 60 | HEX | Auxin efflux facilitator isoform 6 [Theobroma cacao] |